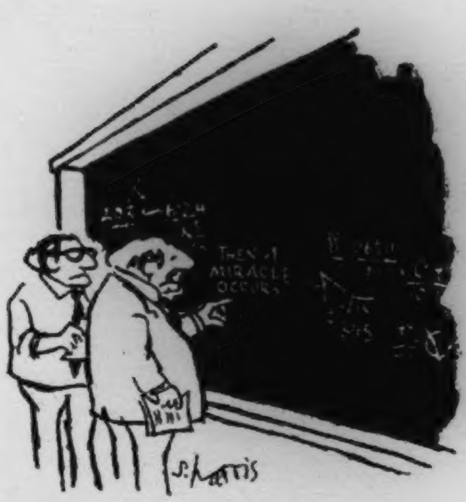
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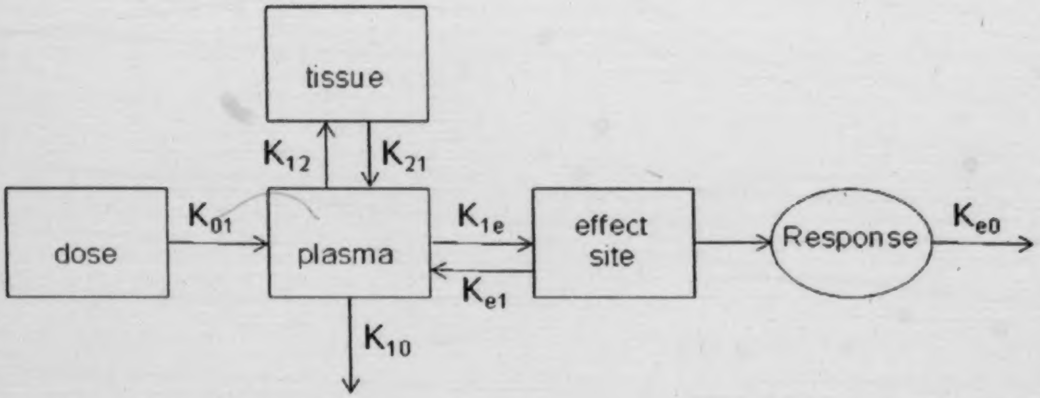
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UNIVERSITY OF ALBERTA

The Effect of Inflammation and Anti-TNFα on Pharmacokinetics and Pharmacodynamics of Verapamil:

A Disease-Drug Interaction

by

SPENCER YU-HONG LING ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Pharmaceutical Sciences

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ABSTRACT

Pharmacokinetics (PK) and pharmacodynamics (PD) of drugs that are efficiently cleared by the liver are altered by inflammation. Elevated pro-inflammatory mediators are associated with increased acute phase proteins and suppression of drug metabolizing enzymes. Inflammation may also cause the down-regulation of cardiac receptors leading to reduced response to cardiovascular drugs such as verapamil.

The objectives of this thesis work were to: 1) determine the impact of cannulation surgery on the stress response in the rat, 2) investigate the use the pre-adjuvant arthritis (pre-AA) as a pain and distress free model of inflammation in the rat, 3) examine the effects of infliximab on verapamil pharmacokinetics and hepatic CYP enzymes, and 4) assess the effects of disease remission on the pharmacokinetics and pharmacodynamics of verapamil in patients with rheumatoid arthritis (RA).

Baseline corticosterone levels were not influenced post-cannulation surgery. Restraint stress resulted in increased corticosterone levels as early as 1 day following surgery, suggesting a rapid recovery of the stress response system.

Pre-AA was marked by elevated concentrations of nitrite, C-reactive protein (CRP) and tumor necrosis factor-alpha (TNFα) while signs or symptoms of arthritic disease were absent. Decreased CYP450 enzymes and increased verapamil plasma protein binding were observed in pre-AA, resulting in significant increases in plasma S-verapamil concentrations after both oral and iv administration. Pre-AA is therefore a suitable model for the study of the effects of

systemic inflammation on pharmacokinetics while avoiding the pain and distress associated with AA.

Infliximab treatment partially reversed inflammation-mediated suppression of CYP450 contents 8 days following single doses of the drug. Plasma protein binding, however, remained elevated; consequently, verapamil concentrations remained increased. Pro-inflammatory mediators were also significantly elevated despite infliximab treatment.

Rheumatoid arthritis patients in disease remission exhibited low levels of nitrite, interleukin-6 (IL-6) and CRP, indicating well controlled inflammation. TNFα, however, was elevated in patients taking infliximab. Nevertheless, disease remission in both patient groups appeared to reverse the effects of inflammation on verapamil pharmacokinetics. A non-significant reduction in PR interval prolongation was observed in patients with controlled RA. PK/PD modeling of verapamil concentration-effect relationship suggested the potency of verapamil to prolong PR interval was inhibited in RA patients compared with healthy volunteers.

This thesis is dedicated to the memory of my mother Winnie,

and to my daughter April and my wife Eva, whose presence is a reminder of the

true purpose of life.

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List of Abbreviations and Symbols

AA	Adjuvant arthritis
AAG	α_1-acid glycoprotein
ACE	Angiotensin converting enzyme
ARBs	Angiotensin receptor blockers
AUC	Area under the curve
β	Elimination rate constant
BSA	Bovine serum albumin
C	Celsius
$CaCl_2$	Calcium chloride
CL	Clearance
CL/F	Oral clearance
cm	Centimeter
Cmax	Maximum drug concentration
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein
Ct	Total concentration
CTLA	Cytotoxic T-lymphocyte antigen
Cu	Unbound concentration
$CuSO_4$	Cupric sulphate
CV	Coefficient of variation
CYP	Cytochrome P450
DMARD	Disease modifying anti-rheumatic drugs
E	Hepatic extraction ratio
EC_{50}	Concentration at which 50% maximum effect is achieved
ECG	Electrocardiogram
ELISA	Enzyme linked immunosorbent assay
Emax	Maximum effect
FAD	Flavin adenine dinucleotide
fu	Fraction unbound
g	Gram
g	Gravities
h	hour
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IFN	Interferon
IgG	Immunoglobulin G

IL	Interleukin
iNOS	Inducible nitric oxide synthase
ip	Intraperitoneal
iv	Intravenous
KCl	Potassium chloride
kg	Kilogram
M	Molar
MDR	Multi-drug resistant
µg	Microgram
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
mL	Milliliter
µL	Microliter
mm	Millimeter
µm	Micrometer
m*M*	Micromolar
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NSAIDs	Nonsteroidal anti-inflammatory drugs
P450	Cytochrome P450
PD	Pharmacodynamic
pg	Picogram
Pgp	P-glycoprotein
PK	Pharmacokinetic
po	Oral
Pre-AA	Pre-adjuvant arthritis
Q	Liver blood flow
r	Pearson correlation coefficient
RA	Rheumatoid arthritis
sc	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
$t_{1/2}$	Elimination half-life
TEA	Triethylamine
Th	T-helper cells
Tmax	Time to reach maximum drug concentration
TNFα	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor receptor
U	Units
V	Volume of distribution
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

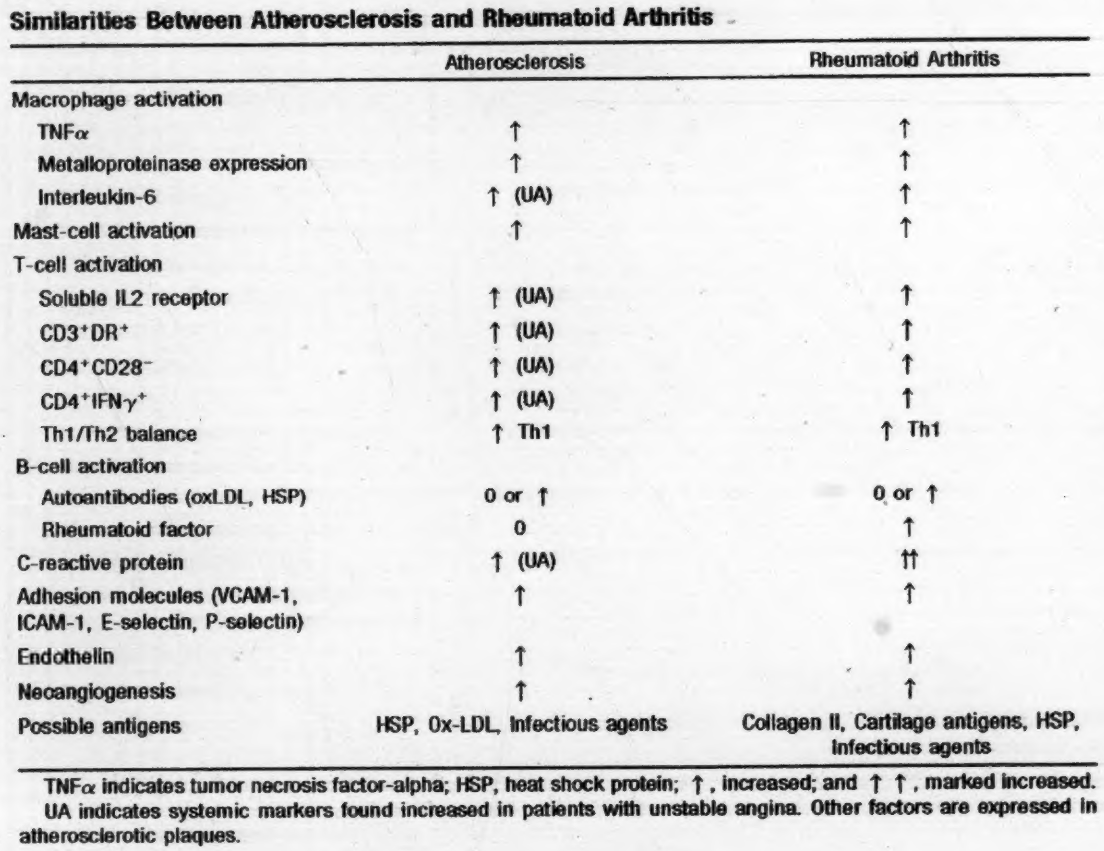
The association between inflammatory status and cardiovascular risk and mortality is well established and extensively documented. Cardiovascular disease is the leading cause of death in Canada (Heart and Stroke Foundation of Canada, 1999) and the United States (Anderson and Smith, 2005) and affects 5% (1 286 000) of Canadians (Chow, et al., 2005). Inflammatory disease is likewise pervasive, affecting nearly 1% of the world population (Harris, et al., 2005). In Canada the prevalence of arthritis is estimated at 16%, and affects nearly 4 million Canadians (Statistics Canada, 2002). Approximately half of all deaths in rheumatoid arthritis (RA) patients are attributable to cardiovascular diseases (Myllykangas-Luosujarvi, et al., 1995) and RA patients show 2-fold increase in mortality rate (Solomon, et al., 2003; Wolfe, et al., 1994) and shorter life span compared with the general population (Myllykangas-Luosujarvi, et al., 1995). While some studies show that cardiovascular deaths in RA patients may be linked to disease severity (Wolfe, et al., 1994), recent evidence also suggests that increased risk of death from heart disease, possibly due to the development of subclinical vascular disease (Alkaabi, et al., 2003), is present in the early years of inflammatory arthritis (Goodson, et al., 2002). Rheumatoid arthritic patients also have a higher prevalence of angina and elevated diastolic blood pressure (McEntegart, et al., 2001).

Much attention has recently been given towards addressing the common pathogenic features of cardiovascular and inflammatory disease such as RA

(Kulmatycki and Jamali, 2001; Kulmatycki and Jamali, 2005; Pasceri and Yeh, 1999; Sattar, et al., 2003). Indeed, cardiovascular diseases are now considered inflammatory conditions. The activation of inflammatory cells and release of pro-inflammatory cytokines that characterize the immune response are implicated in the pathogenesis of atherosclerosis (Table 1-1). Cardiovascular conditions including atherosclerosis, myocardial infarction, heart failure, and hypertension are all associated with pro-inflammatory mediators (Cesari, et al., 2003; Peeters, et al., 2001; Ridker, et al., 2000) and are the predominant cause of mortality in patients with RA (Myllykangas-Luosujarvi, et al., 1995; Wolfe, et al., 1994).

The presence of pro-inflammatory mediators is associated with elevated plasma drug concentrations of some cardiovascular drugs in patients with RA (Mayo, et al., 2000) as well as in various animal models of inflammation (Guirguis and Jamali, 2003; Sattari, et al., 2003). Reduced clearance of propranolol and verapamil may result from inflammation-induced increases in protein binding and inhibition of hepatic drug metabolizing enzyme activity (Mayo, et al., 2000; Schneider, et al., 1981). The consequence of elevated drug concentrations is generally increased pharmacologic activity or drug toxicity, however, in patients with RA, increased concentrations of verapamil result in an unexpected reduction in potency (Mayo, et al., 2000). Decreased pharmacodynamic response is also reported for propranolol and sotalol, perhaps as a consequence of receptor down-regulation, and/or altered receptor signaling (Guirguis and Jamali, 2003; Kulmatycki, et al., 2001). Reduced cardiovascular sensitivity may therefore contribute, in part, to the increased susceptibility of

Table 1-1.



Similarities Between Atherosclerosis and Rheumatoid Arthritis

	Atherosclerosis	Rheumatoid Arthritis
Macrophage activation		
TNFα	↑	↑
Metalloproteinase expression	↑	↑
Interleukin-6	↑ (UA)	↑
Mast-cell activation	↑	↑
T-cell activation		
Soluble IL2 receptor	↑ (UA)	↑
CD3$^+$DR$^+$	↑ (UA)	↑
CD4$^+$CD28$^-$	↑ (UA)	↑
CD4$^+$IFN-γ^+	↑ (UA)	↑
Th1/Th2 balance	↑ Th1	↑ Th1
B-cell activation		
Autoantibodies (oxLDL, HSP)	0 or ↑	0 or ↑
Rheumatoid factor	0	↑
C-reactive protein	↑ (UA)	↑↑
Adhesion molecules (VCAM-1, ICAM-1, E-selectin, P-selectin)	↑	↑
Endothelin	↑	↑
Neoangiogenesis	↑	↑
Possible antigens	HSP, Ox-LDL, Infectious agents	Collagen II, Cartilage antigens, HSP, Infectious agents

TNFα indicates tumor necrosis factor-alpha; HSP, heat shock protein; ↑, increased; and ↑ ↑, marked increased. UA indicates systemic markers found increased in patients with unstable angina. Other factors are expressed in atherosclerotic plaques.

Reproduced from Pasceri V and Yeh ET (1999) A tale of two diseases: atherosclerosis and rheumatoid arthritis. *Circulation* **100:**2124-2126.

patients with inflammatory diseases to the development and progression of cardiovascular disease. If this is the case, then one may ask whether the reduction of inflammation with novel anti-inflammatory drugs would reverse the suppressive effects of inflammation on drug potency and lead to improved cardiovascular outcome in this patient group.

It is of interest to note that several classes of cardiovascular drugs have proven unexpectedly beneficial for reducing cardiovascular morbidity and mortality as a result of anti-inflammatory mechanisms unrelated to the drugs' primary mode of action. Drugs such as the cholesterol lowering statins and antihypertensive angiotensin converting enzyme (ACE) inhibitors have demonstrated benefits in regards to cardiovascular morbidity and mortality beyond their ability to lower cholesterol and blood pressure, respectively (Dagenais and Jamali, 2005; Schonbeck and Libby, 2004). These drugs exhibit anti-inflammatory properties with protective effects on the vascular and immune systems. Traditional anti-inflammatory therapies such as disease modifying anti-rheumatic drugs (DMARDs) and the new biologic response modifiers (biologics) are offering new hope to patients suffering from inflammatory diseases such as RA and Crohn's Disease. Emerging evidence now suggest that effective treatment of inflammation may also lead to improvements in cardiovascular outcome (Choi, et al., 2002; Jacobsson, et al., 2005; Krishnan, et al., 2004).

As inflammation is linked to a number of key factors that lead to physiologic changes that may result in altered plasma drug concentrations and reduced drug response, we wanted to study the effect of anti-inflammatory therapy with a tumor necrosis factor-alpha (TNFα) blocking drug on pharmacokinetics and metabolism in the rat and on pharmacokinetics and pharmacodynamics in humans.

In order to carry out our investigations in the rat, we first had to resolve two methodological issues: 1) whether the surgical procedure of cannulation is a significant source of stress which is strong enough to interfere with subsequent stress responses and, 2) to develop an animal model with sustained inflammation that would allow for a study of the effect of anti-TNF. Once these objectives were resolved, we then examined the influence of anti-TNFα on hepatic CYP content and pharmacokinetics of the cardiovascular drug verapamil in the rat and on the pharmacokinetic and pharmacodynamics of verapamil in patients with RA.

Stress

Physiologic response to stress involves activation of the endocrine and sympathetic nervous systems. Stimulation of neurohormonal pathways leads to enhanced adrenal secretion of catecholamines (epinephrine and norepinephrine), hypothalamic release of corticotropin releasing hormone, and pituitary secretion of adrenocorticotropic hormone and arginine vasopressin (Sapolsky, et al., 2000). The release of these hormones results in increased cardiovascular tone, enhancing cardiac output and redistribution of organ perfusion (Blanc, et al., 1991; Freyschuss, et al., 1988; Linde, et al., 1989; Pollack, et al., 1991), all of which allow one to cope with a threat or stressor.

The generalized stress response causes changes in physiologic functions that influence various aspects of drug pharmacokinetics. For some drugs, oral absorption has been shown to be reduced in animal models of stress (Gomita, et al., 1989a; Gomita, et al., 1989b; Okazaki, et al., 1995) due perhaps to alterations in gastric motility (Kennedy and Riji, 1998) and mucosal blood flow (Lockenvitz, et al., 1983). Stress-induced increase in drug tissue distribution has also been reported. The anticonvulsant action of zonisamide was significantly enhanced by immobilization stress in mice despite lower serum concentrations due to an apparent increase in uptake or distribution of zonisamide into the brain (Hashimoto, et al., 2001). The tissue distribution of protein bound drugs may also be affected by stress-induced changes in albumin and α_1-acid glycoprotein concentrations in plasma (Maes, et al., 1997b). Increased plasma proteins are also found in inflammatory conditions and may reflect the complex relationship between the neuro-hormonal and immune systems (Liao, et al., 1995; Zhou, et al., 1993). Animal models of stress have shown decreased levels of cytochrome P450 and reduced activity of some CYP isoenzymes in mice (Konstandi, et al., 1998). Non-traumatic stress has been shown to decrease the clearance of antipyrine, suggesting inhibition of hepatic oxidation, and reduce the clearance of indocyanine green, a marker of hepatic uptake and biliary excretion (Pollack, et al., 1991). The mechanism by which stress alters drug metabolizing enzymes is not clear. Enzyme inhibition may be altered directly by stress hormones or mediated via stress-induced increases in hepatic cytokine production (Black and Garbutt, 2002; Konstandi, et al., 1998). The regulation of the stress and immune systems is complex, as illustrated in Figure 1-1, which depicts the many pathways by which the stress and immune functions can influence the development of atherosclerosis (Black and Garbutt, 2002). Emotional and physical stress has been associated with coronary heart disease, hypertension, hyperlipidemia, and reduced glycemic control (Alexander, et al., 1996; Lloyd, et al., 1999; Rozanski, et al., 1999; Stoney, et al., 1999).

The stress system interacts with the immune system to promote or inhibit inflammatory responses (Elenkov and Chrousos, 2002). The specific pro- or anti-inflammatory response elicited via the neurohormonal pathway depends on the nature and intensity of the stressor. It is generally viewed that the products of hypothalamic-pituitary-adrenal axis and sympathetic nervous system activation are glucocorticoids and catecholamines. Glucocorticoids are potently anti-inflammatory through suppression of TNFα, interferon (IFN)γ and interleukin (IL)-2. Glucocorticoids are also potent inhibitors of IL-12, a key cytokine involved in IFNγ synthesis and inhibition IL-4 production by T cells; thus shifting the Th1/Th2 balance towards an anti-inflammatory Th2 profile (Figure 1-2). Catecholamines also promote a Th2 shift via suppression of the pro-inflammatory Th1 cytokine IL-12 and by upregulating the production of the anti-inflammatory cytokine IL-10 by monocytes/macrophages (Figure 1-2). As the stress system is stimulated by immune cell activation of cytokines such as TNFα, IL-1 and IL-6, it appears that the stress system acts as an important negative feedback mechanism, protecting the host from excessive systemic pro-inflammatory activity. This is supported by findings that chronic stress is associated with suppressive effects on

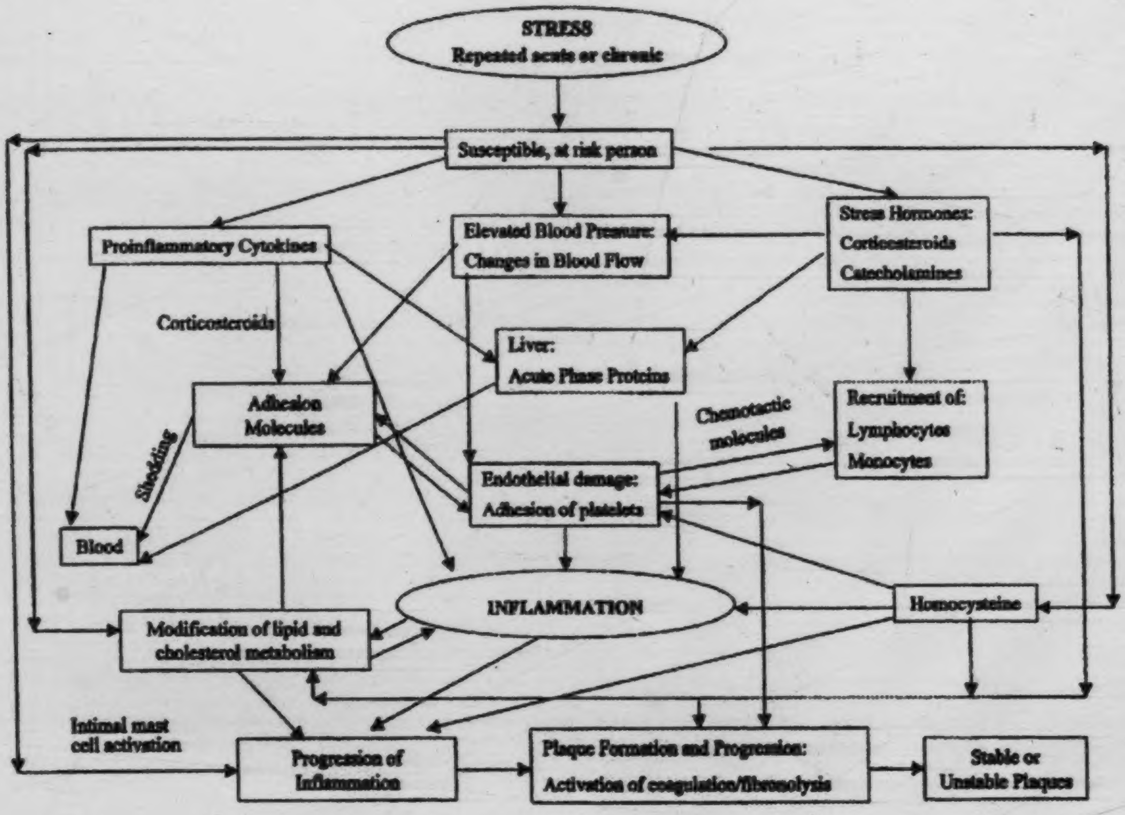

STRESS
Repeated acute or chronic
Susceptible, at risk person
Proinflammatory Cytokines
Elevated Blood Pressure:
Changes in Blood Flow
Stress Hormones:
Corticosteroids
Catecholamines
Corticosteroids
Liver:
Acute Phase Proteins
Adhesion
Molecules
Shedding
Chemotactic
molecules
Recruitment of:
Lymphocytes
Monocytes
Endothelial damage:
Adhesion of platelets
Blood
INFLAMMATION
Homocysteine
Modification of lipid and
cholesterol metabolism
Intimal mast
cell activation
Progression of
Inflammation
Plaque Formation and Progression:
Activation of coagulation/fibrenolysis
Stable or
Unstable Plaques

Figure 1-1. Stress, inflammation, and atherosclerosis. Reproduced from Black PH and Garbutt LD (2002) Stress, inflammation and cardiovascular disease. *J Psychosom Res* **52**:1-23.

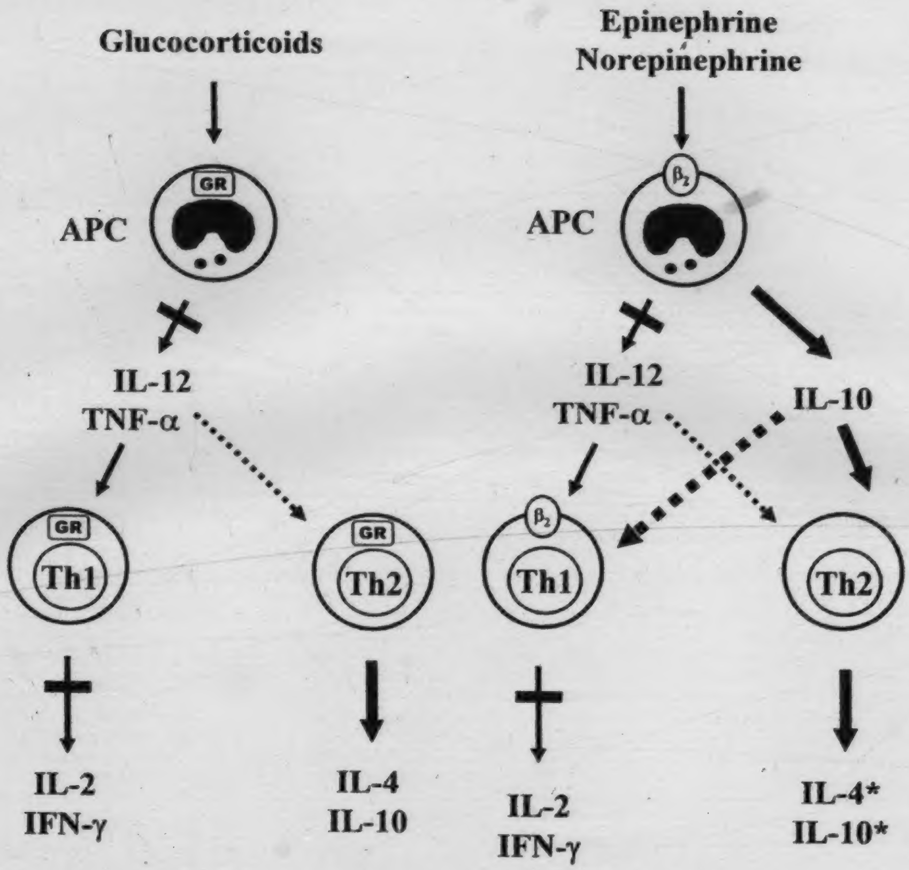

Glucocorticoids
Epinephrine
Norepinephrine
GR
β_2
APC
APC
IL-12
TNF-α
IL-12
TNF-α
IL-10
GR
Th1
GR
Th2
β_2
Th1
Th2
IL-2
IFN-γ
IL-4
IL-10
IL-2
IFN-γ
IL-4*
IL-10*

Figure 1-2. Systemic effects of stress hormones on pro-inflammatory and anti-inflammatory cytokine production. Solid lines represent stimulation; dashed lines represent inhibition. Abbreviations: APC, antigen-presenting cell; β, β-adrenergic receptor; GR, glucocorticoid receptor; IL, interleukin; IFN, interferon; Th, T helper lymphocyte; TNF, tumor necrosis factor. Reproduced from Elenkov IJ and Chrousos GP (2002) Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann N Y Acad Sci* **966**:290-303.

the immune system and that inflammatory autoimmune disease is in part facilitated by a hypoactive stress system characterized by deficient cortisol production (Elenkov and Chrousos, 2002; Walker, et al., 1999). Acute stressors, conversely, are associated with increases in cortisol and catecholamines, although this may be a function of specific circumstances. In RA patients for example, stress has been shown to suppress disease activity through cortisol and catecholamine suppression of Th1 activity (Affleck, et al., 1987), and yet, has also been implicated by patients as the most common cause for disease flare-ups (Zautra, et al., 1989) perhaps as a consequence of a homeostatic imbalance. Psychological stress in humans such as short term acute stress caused by exam anxiety increased the production of Th1 cytokines TNFα, IL-6, IL-1 receptor antagonist (IL-1Ra), IFNγ and IL-10 (Maes, et al., 1998) and increased expression of neutrophils, monocytes, and CD8+ cells (Maes, et al., 1999). Perception of high stress level was also related to higher inflammatory cytokine levels than those with lower perceived stress level. In addition, students with a high anxiety response had a lower production of the negative immunoregulatory cytokines, IL-10 and IL-4, than students without anxiety. Post-traumatic stress disorder has been associated with increased IL-6 signaling (Maes, et al., 1999). The stress system clearly plays a homeostatic role in regulating immune responses, at times enhancing and at other times diminishing inflammatory processes. It is not known whether the stress and inflammation caused by cannulation surgery is of an intensity and nature sufficient to alter subsequent stress responses and therefore potentially pharmacokinetics and pharmacodynamics in the post-surgical rat.

Inflammation

Inflammation is a physiologic response to tissue injury and infection (Kuby, 1997). A localized acute inflammatory reaction is characterized by redness, pain, heat and swelling at the inflammatory site due to increased blood volume, leakage of fluid from blood vessels and accumulation of blood cells into the affected tissues (Figure 1-3). Vascular injury causes the release of bradykinin and fibrinopeptides and activates complement-induced release of histamine, prostaglandins and leukotrienes from mast cells; all of which induce vasodilation and increase vascular permeability. In addition, damaged vascular endothelial cells express cell adhesion molecules, such as E- and P-selectin, that attracts and binds to leukocytes. First neutrophils and then monocytes and macrophages infiltrate inflamed tissue where they phagocytose invading pathogens. Importantly, activated macrophages secrete IL-1, IL-6, and TNFα, cytokines with similar pleiotropic and pro-inflammatory functions (Table 1-2). All three cytokines also induce a systemic inflammatory response known as an *acute phase response*. This is a generalized response characterized by fever, increased synthesis of adrenal hormones, increased production of leukocytes and increased production of acute phase proteins by the liver.

Persistent immune activation leads to chronic inflammation (Kuby, 1997). While an acute inflammatory response is essential for immune defense, chronic inflammation can result in tissue damage and may by an underlying factor in cardiovascular disease, wasting syndromes associated with cancer, and autoimmune diseases. Two key features of chronic inflammation are the

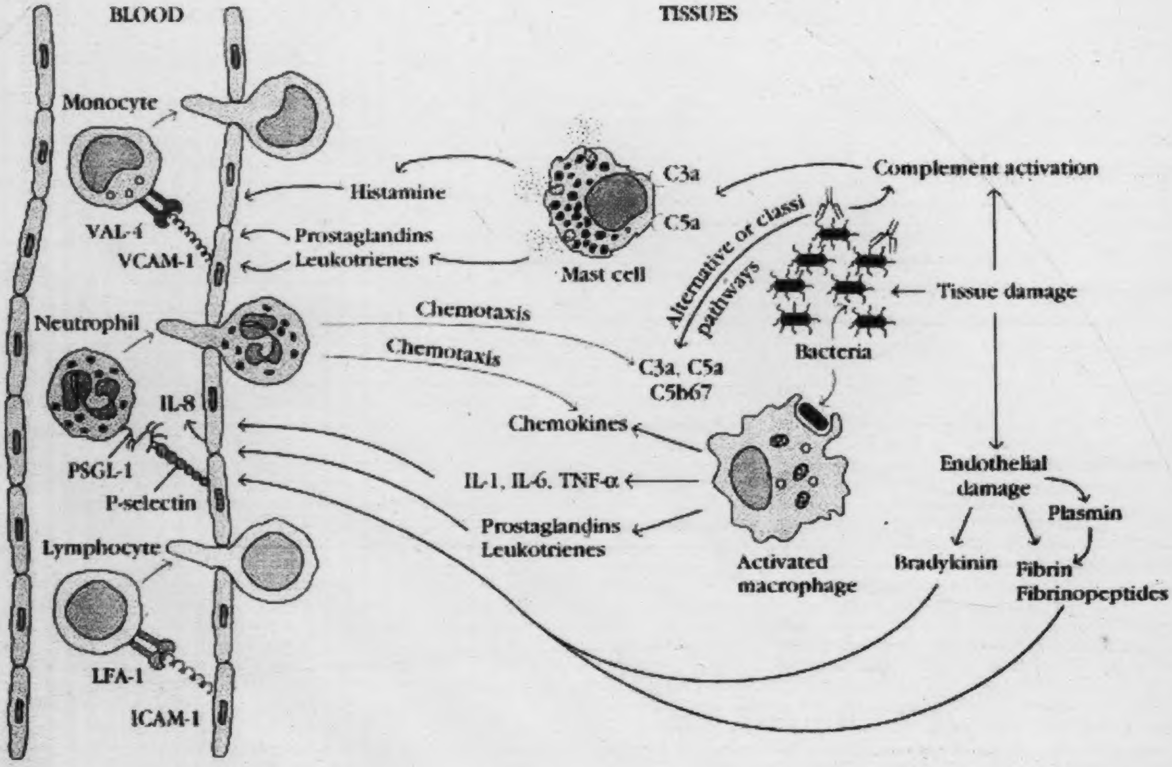

BLOOD
TISSUES
Monocyte
VAL-4
VCAM-1
Histamine
Prostaglandins
Leukotrienes
Mast cell
C3a
C5a
Complement activation
Alternative or classi pathways
Tissue damage
Bacteria
Neutrophil
Chemotaxis
Chemotaxis
C3a, C5a
C5b67
IL-8
PSGL-1
P-selectin
Chemokines
IL-1, IL-6, TNF-α
Prostaglandins
Leukotrienes
Activated
macrophage
Endothelial
damage
Plasmin
Bradykinin
Fibrin
Fibrinopeptides
Lymphocyte
LFA-1
ICAM-1

Figure 1-3. The local acute inflammatory response. Reproduced from Kuby J (1997) *Immunology.* W.H. Freeman, New York. Leukocyte migration and inflammation, Ch 15.

Table 1-2. Redundant and Pleiotropic Effects of IL-1, TNF-α, and IL-6. Reproduced from Kuby J (1997) *Immunology*. W.H. Freeman, New York. Leukocyte migration and inflammation, Ch 15.

Effect	IL-1	TNF-α	IL-6
Endogenous pyrogen fever	+	+	+
Synthesis of acute-phase proteins by liver	+	+	+
Increased vascular permeability	+	+	+
Increased adhesion molecules on vascular endothelium	+	+	−
Fibroblast proliferation	+	+	−
Platelet production	+	−	+
Chemokine induction (e.g., IL-8)	+	+	−
Induction of IL-6	+	+	−
T-cell activation	+	+	+
B-cell activation	+	+	+
Increased immunoglobulin synthesis	−	−	+

accumulation of activated macrophages and T cells and increased secretion of cytokines. Macrophages, along with B lymphocytes and dendritic cells, function as antigen presenting cells – the primary activators of the adaptive arms of the immune system. Antigen presenting cells present foreign pathogens (or autoantigens in the case of autoimmune disorders) to T-lymphocytes. The subsequent differentiation and proliferation of T helper (Th) cells into one of two subsets determines the specific immune response elicited (Figure 1-4). T helper type 1 (Th1) lymphocytes favor cell-mediated functions whereas Th2 cells function primarily as helpers for B-cell activation. The activation of Th1 cells stimulates the release of pro-inflammatory cytokines (IL-2, IFNγ, TNFβ and granulocyte macrophage-colony stimulating factor) and proliferation of Th1 cells. Th2 cell proliferation tends to promote anti-inflammatory cytokine release (IL-4, IL-5 and IL-10) and proliferation of Th2 response. Both subtypes of T-cells mutually suppress the proliferation of the other subset, thereby promoting the dominance of one type of inflammatory response over another. It is believed that the balance of Th1/Th2 cells determines the course of inflammatory disease. For example, autoimmune diseases such as RA and Crohn's disease are considered Th1 dominant conditions. Th1-mediated inflammation has also been implicated in the development of atherosclerosis and modulation of Th1/Th2 balance by angiotensin receptor blocking drugs and statins may be responsible in part for the beneficial effects of these drugs in reducing cardiovascular disease (Dagenais and Jamali, 2005; Hakamada-Taguchi, et al., 2003). Pregnancy has been associated

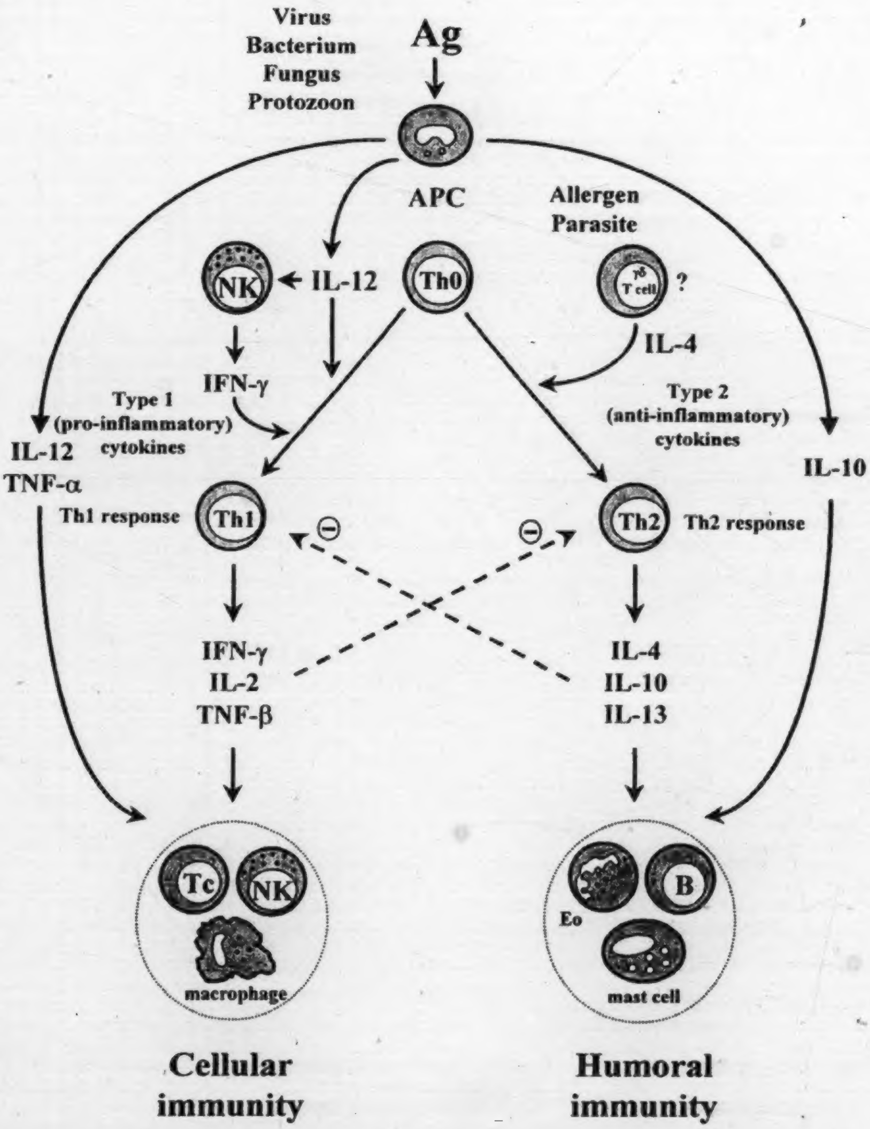

Virus
Bacterium
Fungus
Protozoon
Ag
APC
Allergen
Parasite
NK
IL-12
Th0
γδ T cell
?
IL-4
IFN-γ
Type 1
(pro-inflammatory)
cytokines
Type 2
(anti-inflammatory)
cytokines
IL-12
TNF-α
IL-10
Th1 response
Th1
Th2
Th2 response
IFN-γ
IL-2
TNF-β
IL-4
IL-10
IL-13
Tc
NK
macrophage
Eo
B
mast cell
Cellular immunity
Humoral immunity

Figure 1-4. Role of APCs, Th1 and Th2 cells, and pro-inflammatory and anti-inflammatory cytokines in the regulation of cellular and humoral immunity. Solid lines represent stimulation; dashed lines represent inhibition. Abbreviations: Ag, antigen; APC, antigen-presenting cell; NK, natural killer cell; B, B cell; Th, T helper cell; Tc, T cytotoxic cell; Eo, eosinophil; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon. Reproduced from Elenkov IJ and Chrousos GP (2002) Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann N Y Acad Sci* **966:**290-303.

with beneficial effects in inflammatory arthritis due to a Th2 predominance (Straub, et al., 2005). Nevertheless, all inflammatory conditions are associated with altered mediators that may influence the pharmacokinetics and pharmacodynamics of drugs.

Rheumatoid arthritis is a chronic inflammatory disease of the joints. It is characterized by persistent inflammation, accumulation of inflammatory cells and dominance of Th1 lymphocytes (Harris, et al., 2005). Activated Th1 cells stimulate macrophages and fibroblasts to produce nitric oxide and matrix metalloproteases (MMP) and pro-inflammatory cytokines. Cytokines promote inflammation and stimulate the expression of chemokines and cell adhesion molecules. Cytokines also stimulate angiogenesis via vascular endothelial growth factor (VEGF) resulting in synovial hyperplasia (Choy and Panayi, 2001). Combined with the leaky vasculature characteristic of inflamed tissue, the RA joint becomes the site of massive accumulation of inflammatory cells and debris known as a pannus. Progressively, the presence of proteases and excess reactive oxygen species in the pannus as well as the activation of osteoclasts lead to erosion and destruction of the cartilage and bone. Although RA affects mostly joints, it can also cause a variety of extra-articular manifestations. Patients with RA have a high level of systemic inflammation marked by elevated serum concentrations acute phase proteins such as α_1-acid glycoprotein (AAG), serum amyloid A and C-reactive protein (CRP). Three key cytokines in the pathogenesis of RA are TNFα, IL-1 and IL-6. Each can be induced by a variety of mechanisms and is pro-inflammatory and are implicated in local and systemic inflammatory response in RA.

Inflammatory Mediators

Tumor Necrosis Factor-α

Tumor necrosis factor alpha (TNFα), named for its antitumour properties and cytotoxicity (MacEwan, 2002), is a pro-inflammatory cytokine secreted mainly by macrophages/monocytes, but also by fibroblasts, mast cells and activated T cells in response to tissue injury or immune stimulation (Choy and Panayi, 2001). TNF is a homotrimer protein expressed on the cell membrane surface (transmembrane TNF) where it may be released via cleavage by TNFα converting enzyme to its soluble form. Both transmembrane and soluble TNF binds to one of two naturally occurring receptors: TNF receptor 1 (TNFR1, also known as p55 receptor) and TNFR2 (p75 receptor). Although both TNFR1 and TNFR2 are capable of signaling apoptosis, most TNF effects *in vivo* are mediated by transmembrane TNF via the TNFR1 receptor (MacEwan, 2002). TNFR1 receptor binding leads to the internalization of the receptor followed by the activation of a series of intracellular signal transduction pathways that stimulate cellular responses, whereas, TNFR2 receptor binding induces shedding of the soluble TNF/TNFR2 complex. The binding of soluble TNF to the soluble receptor serves as a regulatory mechanism for TNF activity. Binding of TNF to the soluble receptor appears to antagonize TNF activity by preventing TNF from binding and activating the more potent TNFR1 receptor, however, due to the reversible nature of the binding, soluble TNFR2 prolongs the half-life of soluble TNF, increasing its local concentration in the vicinity of TNFR1 receptors, which can later, accept TNF from the TNFR2 receptor and itself become activated (MacEwan, 2002). TNF receptors are expressed on many cell types including those of the synovial membrane and cartilage-pannus junction (Deleuran, et al., 1992). In fact, high levels of soluble TNF receptors are found in the serum and synovial fluid of patients with RA (Cope, et al., 1992) and in the failing myocardium of patients with congestive heart failure (Torre-Amione, et al., 1999). The concentrations of receptors however, are insufficient to inactivate the overproduction of TNFα under these inflammatory conditions (Choy and Panayi, 2001).

TNF exerts a number of effects that contribute to the inflammatory cascade and immune activation. TNF induces the production of other pro-inflammatory cytokines such as IL-1, IL-6, acute phase proteins and prostaglandin E_2 (Feghali and Wright, 1997). Other pro-inflammatory effects include the stimulation of fibroblasts to express adhesion molecules such as intercellular adhesion molecule 1. Adhesion molecules increase the migration of leucocytes into inflammatory sites such as the joints of patients with RA (Choy and Panayi, 2001) and atherosclerotics plaques of vascular walls (Pasceri and Yeh, 1999). TNF stimulates angiogenesis via VEGF, a process known to contribute to the development of both RA and atherosclerosis (Pasceri and Yeh, 1999). Additional pro-inflammatory effects of TNF central to the pathogenesis of rheumatoid arthritis are the stimulation of MMPs and osteoclasts. Over expression of these immunoreactive proteins and cells in the synovium leads to joint inflammation and destruction (Choy and Panayi, 2001; Feghali and Wright, 1997). Elevated levels of TNFα have been found in serum and synovial fluid of rheumatoid arthritis patients and correlated with disease activity (Manicourt, et al., 1993; Saxne, et al., 1988). Blockade of TNFα with TNF antagonists has been shown to decrease pain and joint swelling, reduce rates of radiologic joint damage, and lead to significant improvements in clinical outcome in patients with RA (Elliott, et al., 1993; Genovese, et al., 2002; Weinblatt, et al., 2003).

Interleukin-1

IL-1 is a key cytokine in the pathogenesis of RA. It is produced by activated macrophages and increased synovial fluid levels of IL-1 in RA patients are correlated with disease activity (Eastgate, et al., 1988). It is also localized in endothelium of atherosclerotic vessels in coronary artery disease (Dewberry, et al., 2000). Pro-inflammatory effects of IL-1 include activation of monocytes/macrophages, hence expression of IL-1 may be induced by pro-inflammatory cytokines such as TNFα as well as by self-induction. Additional pro-inflammatory effects include the stimulation of prostaglandin E_2, nitric oxide, and MMPs. In animal studies, direct injection of IL-1 into joints causes cartilage damage (Chandrasekhar, et al., 1990), perhaps through activation of chondrocytes and osteoclasts resulting in cartilage and bone destruction and resorption and inhibition of synthesis of collagen and proteoglycan (Bresnihan, et al., 1998). In coronary artery vessels, elevated IL-1 stimulates activity of MMPs which contribute to the destabilization and rupture of atherosclerotic plaques (Rajavashisth, et al., 1999; Ruhul Amin, et al., 2003). The IL-1 gene family includes IL-1α and IL-1β as well as IL-1 receptor antagonist (IL-1ra) (Arend, 1991). IL-1α and IL-1β are the membrane bound and soluble agonists that activate target cells by binding to the IL-1 receptor (IL-1r). The IL-1ra is a naturally occurring inhibitor of IL-1 that competitively binds to the IL-1 receptor and acts as a regulator of IL-1 function. IL-1ra are also produced by RA synovial tissue and coronary vessel endothelium, although production of IL-1ra appears to be deficient compared with IL-1 production in inflammatory disease (Choy and Panayi, 2001; Dewberry, et al., 2000).

Interleukin-6

IL-6 is a pleiotropic cytokine that is both a marker and mediator of inflammatory responses. IL-6 also has regulatory functions in immune processes through its effects on a wide range of target cells (Feghali and Wright, 1997; Nishimoto, 2005). Pro-inflammatory actions of IL-6 include the stimulation of the acute phase response and promotion of B-cell maturation and activation of T cells. Over-production of IL-6 may induce the expression of IL-2 and adhesion molecules resulting in the proliferation and accumulation of activated immune cells. IL-6 stimulates the production of platelets and acts synergistically with TNFα and IL-1 to stimulate production of vascular endothelial growth factor, a potent inducer of angiogenesis. Angiogenesis is implicated in vascular endothelial dysfunction as well as synovial tissue hyperplasia. Furthermore, in RA, IL-6 induces osteoclast maturation contributing to bone and cartilage destruction and osteoporosis. The regulatory effects of IL-6 include apparently anti-inflammatory functions. For example, IL-6 has been shown to inhibit the production of TNF and IL-1 and induce the release of IL-1ra (Schindler, et al., 1990). Nevertheless, IL-6 has been implicated in the pathogenesis of inflammation-mediated diseases such as RA, coronary artery disease, and Crohn's disease (Lindmark, et al., 2001; Manicourt, et al., 1993; Nishimoto, 2005).

C-reactive Protein

The first acute phase reactant to be identified was C-reactive protein (CRP) (Pepys and Hirschfield, 2003). In healthy individuals, serum levels of this protein are low, usually in the range of ~1.0 μg/ml but can increase to more than 500 μg/ml, or 10,000-fold, by an acute-phase stimulus. CRP is primarily produced by hepatocytes, under the regulation of pro-inflammatory cytokines IL-6, IL-1β, TNFα, IFNγ, and transforming growth factor β (Gabay and Kushner, 1999). Upon inflammatory stimulus, CRP synthesis begins rapidly, with serum concentrations rising above 5 μg/ml by 6 h and peaking around 48 hours. The plasma half-life is about 19 h, and is constant under all conditions (Vigushin, et al., 1993). Plasma concentrations of CRP are therefore determined by the synthesis rate and thus reflect the intensity of pathological processes that stimulate CRP production. CRP is a sensitive and non-specific marker of inflammation as seen by high levels in patients with RA (Charles, et al., 1999; Daneshtalab, et al., 2004) and coronary heart disease (Cushman, et al., 2005). In addition, CRP may have a role as a pro-inflammatory mediator. After binding to various target ligands, CRP activates the classical complement pathway, a function similar to antibodies, suggesting possible CRP contribution to host defense against infection (Pepys and Hirschfield, 2003). In support of this theory is the wide range of known molecules to which CRP binds, including autologous ligands such as lipoproteins, phospholipids and damaged cell membranes, and extrinsic ligands including many glycan, phospholipid, and other constituents of microorganisms, such as capsular and somatic components of bacteria, fungi, and parasites, as well as plant products (Pepys and Hirschfield, 2003). In coronary artery disease, CRP has been found to bind selectively to oxidized and enzyme-modified low density lipoproteins as found in atheromatous plaques (de Beer, et al., 1982). That CRP was found deposited in a majority of such plaques supports the role of CRP in atherogenesis (Zhang, et al., 1999). In patients with cardiovascular disease, even small elevations in CRP concentrations are reflective of underlying inflammation and are predictive of future cardiovascular events. For example, CRP has been associated with poorer outcomes for patients with unstable angina (Verheggen, et al., 1999) and post-myocardial infarction (Pietila, et al., 1996) as well as the presence and progression of atherosclerosis (Gonzalez-Gay, et al., 2005; Tzoulaki, et al., 2005). Pro-atherogenic effects of CRP have been demonstrated in human endothelial cells where it increases expression of adhesion molecules and chemokines (Pasceri, et al., 2000; Pasceri, et al., 2001). CRP may also be involved in cardiomyopathies as suggested by experiments that reported increases in IL-1β-induced expression of iNOS and increased NO production in rat cardiac myocytes (Ikeda, et al., 2002).

Nitric Oxide

Nitric oxide (NO) is a vasodilatory molecule produced constitutively by nitric oxide synthase (NOS) in endothelial and neuronal tissue by eNOS and nNOS, respectively (Moncada, et al., 1991). NO exerts its effects through guanylyl cyclase to increase cyclic guanosine monophosphate (cGMP) production resulting in reduced intracellular calcium which in turn leads to diminished smooth muscle activity (Moncada, et al., 1991). At physiological concentrations, NO mediates protective functions of the endothelium by inhibiting vascular recruitment of leukocytes, platelet adhesion and smooth muscle proliferation and is a key factor in the maintenance of vascular tone (Ganz and Vita, 2003). Endothelial dysfunction by contrast is characterized by impaired eNOS-mediated NO production and is a strong predictor of cardiovascular disease (Ganz and Vita, 2003; Verma, et al., 2002).

Under inflammatory conditions, cytokines stimulate the production of high levels of NO through inducible nitric oxide synthase (iNOS) (Moncada, et al., 1991). The large amounts of NO produced under these conditions play an important role in host immune defense. For example, immune stimulation induces macrophage NOS to produce large amounts of NO; the effector molecule for activated macrophage-induced cytotoxicity (Hibbs, et al., 1988). Overproduction of NO however, has been linked to inflammatory diseases such as rheumatoid arthritis and heart failure (Sawyer and Colucci, 1998; Ueki, et al., 1996). In addition to direct toxic effects, NO can at high concentrations react with oxygen to form highly toxic reactive oxygen species. Oxidative stress also results from reaction of NO with other oxygen species such as the super oxide anion (O_2^-) to form peroxynitrite ($ONOO^-$) (Wink, et al., 1996). High concentrations of NO therefore has deleterious effects on the cardiovascular system causing vascular damage and impaired myocardial function (Sawyer and Colucci, 1998). In RA, over production of NO mediates joint and cartilage damage via activation of metalloproteases, suppression of collagen and proteoglycan synthesis and up-regulation of pro-inflammatory cytokine production (Vuolteenaho, et al., 2002). Increased NO is found in the serum and inflamed joints of RA patients where levels are correlated with disease severity (Ueki, et al., 1996). Inhibition of NO production by iNOS inhibitors has been shown to ameliorate NO-mediated oxidative damage to cardiac myocytes (Pinsky, et al., 1995) and synovial tissue damage and cartilage degradation in animal models of arthritis (Connor, et al., 1995; McCartney-Francis, et al., 1993).

Anti-Inflammatory Therapies

Arthritis and related conditions are costly illnesses, as musculoskeletal disorders account for greater than 10% of all health care costs in Canada (Badley, 1995). It is estimated that up to 24% of total medical costs associated with rheumatoid arthritis are for medications (Cooper, 2000). The complex immune and inflammatory processes involved in autoimmune diseases such as RA presents both challenges and opportunities for the treatment of such conditions as numerous signaling pathways and cellular targets are available as potential therapeutic targets. The mainstays of arthritis therapy have traditionally been corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs).

Corticosteroids inhibit the metabolism of arachadonic acid, thus blocking the synthesis of pro-inflammatory prostaglandins and leukotrienes. Corticosteroids also suppress the proliferation and activation of a number of immune cells including macrophages and T lymphocytes thus inhibiting the production of Th1 cytokines such as IL-1β, IL-2, IL-3, IL-6, TNF-α, IFNγ, and GMC-SF (Harris, et al., 2005). Corticosteroids are therefore very potent anti-inflammatory agents, but due to their diverse actions, also cause significant adverse effects including osteoporosis, hypertension, and hyperlipidemia (O'Dell, 2004).

NSAIDs inhibit the production of prostaglandins by blocking the cyclooxygenase (COX)-1 and -2 enzymes and are associated with serious gastrointestinal adverse effects (Singh, 1998). The introduction of the COX-2 selective enzyme inhibitors a few years ago promised to revolutionize the treatment of inflammatory disease. By selectively blocking the COX-2 mediated production of inflammatory prostaglandins, these agents reduced inflammation while avoiding the gastrointestinal toxicity attributed to the antagonism of the COX-1 enzyme. Although these drugs have been shown to reduce the incidence of gastric and duodenal ulcers, their efficacy was no better than traditional NSAIDs. Furthermore, both traditional NSAIDs and COX-2 inhibitors have been associated with increased fluid retention, exacerbation of hypertension, and impairment of renal function (O'Dell, 2004). Increased thrombotic events were reported in patients who were taking COX-2 inhibitors compared with those taking placebo or traditional NSAIDs and in 2004, the widely popular COX-2 inhibitor rofecoxib was withdrawn from the market because of evidence of excess cardiovascular events in patients taking rofecoxib compared with patients taking naproxen (Bresalier, et al., 2005; Graham, et al., 2005). Subsequent studies suggested other COX-2 selective inhibitors parecoxib, valdecoxib and celecoxib also potentially carried this risk (Nussmeier, et al., 2005; Solomon, et al., 2005). Clearly, for RA patients, in whom the risk of cardiovascular complications is elevated, the potential for NSAIDs and corticosteroid to negatively affect cardiovascular outcome limit their use.

Importantly, neither corticosteroids nor NSAIDs halts the progression of disease that characterizes RA. Disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate, hydroxychloroquine, gold salts, and leflunomide have varied mechanisms of action though all slow the progression of rheumatic disease. At one time, these agents were introduced to patients only as their disease progressed, but recent changes in clinical practice have lead to the earlier use of DMARDs for reducing inflammation and preventing disease progression (Ward, 1999). In fact, the initiation of DMARD therapy within three months of the diagnosis of RA is recommended; delaying the introduction of these medications by as little as three months results in substantially more radiographic damage at five years (Egsmose, et al., 1995; Lard, et al., 2001; Tsakonas, et al., 2000). This shift toward more extensive use of these agents may be responsible for the observation that fewer RA deaths have been seen in the last decade due to more aggressive anti-inflammatory therapy (Krishnan, et al., 2004).

Cardiovascular Drugs

Statins

Evidence of the role of inflammatory mediators on cardiovascular disease has been demonstrated by evidence of improved cardiovascular outcomes in patients taking statins (Schonbeck and Libby, 2004). Several clinical studies have established cardiovascular benefit of statin therapy (Anonymous 1994; Pedersen, et al., 2004) and moreover, that the benefits were greater than can be attributed to their cholesterol lowering effects (Heart Protection Study Collaborative Group., 2002; Sacks, et al., 1998; Schwartz, et al., 2001). It appears that pleiotropic anti-inflammatory and antioxidant activity of statins (Landmesser, et al., 2005) lower cardiovascular risk by modulating immune and inflammatory mechanisms. Anti-inflammatory effects of statins include the lowering of pro-inflammatory marker levels, such as CRP, IL-6 and TNFα (Schonbeck and Libby, 2004). In vitro studies show statins have anti-inflammatory actions through several mechanisms including inhibition of endothelial adhesion and migration of leukocytes (Weitz-Schmidt, 2002), modulation of macrophage functions by inhibiting the activation of inflammatory response genes, such IL-1β and IL-6, TNFα, MMP-2, and MMP-9, and iNOS (Takemoto and Liao, 2001). In addition, statins may downregulate the activation of transcription factors such as nuclear factor-κB (NF-κβ) (Dichtl, et al., 2003). NF- κB regulates the transcription of many genes, including cytokines, chemokines, adhesion molecules, and growth factors. The anti-inflammatory role of statin therapy is further supported by beneficial effects in immune and inflammatory diseases such as multiple sclerosis, Alzheimer's disease, ischemic stroke, transplant rejection, rheumatoid arthritis, and asthma (Maggard, et al., 1998; McCarey, et al., 2004; McKay, et al., 2004; Stuve, et al., 2003).

ACE Inhibitors/Angiotensin Receptor Blockers

The anti-inflammatory effects of angiotensin II disruption has recently been reviewed by Dagenais and Jamali (Dagenais and Jamali, 2005). Originally introduced as antihypertensive medications, ACE inhibitors and angiotensin receptor blockers (ARBs) have been proven to confer cardiovascular protection beyond their ability to reduce blood pressure. Both have shown significant benefit in reducing mortality in patients with heart failure (Garg and Yusuf, 1995; Maggioni, et al., 2002; Yusuf, et al., 2003), preventing diabetic nephropathy (Capes, et al., 2000; Lewis, et al., 2001) and reducing ischemia in cardiovascular disease (Kondo, et al., 2003; Yusuf, et al., 2000). The renin-angiotensin-aldosterone system regulates the secretion and retention of water and sodium, thus influencing blood pressure. By inhibiting the conversion of angiotensin I to angiotensin II via suppression of ACE, angiotensin II mediated sodium and water retention is reduced, resulting in lower blood pressure. Angiotensin II however, is also a pro-inflammatory molecule. Increasing evidence suggests angiotensin II increases oxidative stress and activates nuclear factor-κB resulting in increased expression of cytokines, adhesion molecules, MMPs, and platelet activator inhibitor-1. The activation of all these immune molecules is believed to contribute to cardiovascular disease. In animal studies, inhibition of angiotensin II activity by ACEI and ARBs reduce NF-κB activation, TNFα and IL-6 production. Human studies have similarly shown anti-inflammatory actions. By modulating the inflammatory effects of angiotensin II, ACEI and ARBs provide vasodilatory and cardiovascular protection.

Biologics

Recently, a new class of drugs called biologic response modifiers, or biologics, has been introduced that represent a major advance for the treatment of rheumatoid arthritis and other inflammatory diseases. Included in this class is the IL-1 receptor antagonist, anakinra, and the TNFα antagonists infliximab, etanercept, and adalimumab. Most recently, a third class of biologic agent has been introduced, a T cell co-stimulation modulator, abatacept. These agents work faster than DMARDs at improving symptoms and preventing disease progression, however, are also very expensive. The annual cost of anti-TNFα therapies is between $15,000 and $20,000 per patient (based on standard doses allowable as benefits under the Alberta Health and Wellness Drug Benefit List).

Interleukin-1 Receptor Antagonist

Interleukin-1 is a key cytokine in RA and coronary artery disease. The IL-1 receptor antagonist is a naturally occurring product of the IL-1 gene family that is anti-inflammatory and thus a regulator of IL-1 function. The IL-1ra is constitutively produced in synovial and coronary vascular endothelium along with IL-1 (Dewberry, et al., 2000; Feldmann and Maini, 1999) and its expression is increased in inflammatory disease (Choy and Panayi, 2001; Dewberry, et al., 2000). In RA however, there is a deficiency of IL-1ra relative to IL-1 due to either inadequate IL-1ra production or over production of IL-1. Human recombinant IL-1ra (anakinra) has been cloned and tested in humans and animals as therapy for RA. Anakinra has been shown to be safe and effective in active and severe RA refractory to DMARD therapy, and although it appears to be less effective than TNF blocking agents, has demonstrated a beneficial effect on the rate of joint erosion (Bresnihan, et al., 1998).

T-cell Co-stimulation Modulator

The presence of activated T cells in sera and joints of RA patients points to the important role of this immune cell in inflammatory disease. Activation of T cells requires two signals: 1) the binding of a T cell receptor with an antigen presented by an major histocompatibility complex (MHC) II molecule on an antigen presenting cell, and 2) binding of adjacent co-stimulatory molecule CD28 to its ligands such as CD80/86 (Kremer, et al., 2003). The presence of both binding signals stimulates the production of cytokines that activate other inflammatory cells and promotes T cell proliferation. A T cell receptor signal in the absence of a co-stimulatory signal results in a poor response and perhaps apoptosis.

Abatacept is the first of a new class of anti-rheumatic biologic therapies that modulate the co-stimulatory signal (Genovese, et al., 2005). Cytotoxic T-lymphocyte antigen 4 (CTLA4) is an inhibitory molecule expressed on the surface of activated T cells. CTLA4 is a naturally occurring receptor for CD80 and CD86 and binds with much greater affinity than CD28. Abatacept is a fusion protein comprised of the extracellular domain of a CTLA4 receptor fused with a human IgG constant region. Abatacept binds both CD80 and CD86 on antigen-presenting cells with higher affinity than CD28, effectively competing with and inhibiting the CD28 generated co-stimulatory signal required for full T cell activation. RA patients treated with abatacept for 6 months demonstrated dose-dependent improvements in tender and swollen joints, pain, and physical function and improved global assessment as well as improvements in CRP levels (Genovese, et al., 2005; Kremer, et al., 2003).

Anti-TNFα

There are currently three TNFα blocking agents available for the treatment of RA: infliximab, etanercept and adalimumab (Figure 1-5). All three drugs are indicated for the treatment of RA and psoriatic arthritis. Infliximab is additionally indicated for Crohn's disease, ulcerative colitis and ankylosing spondylitis whereas etanercept has additional indications for ankylosing spondylitis and

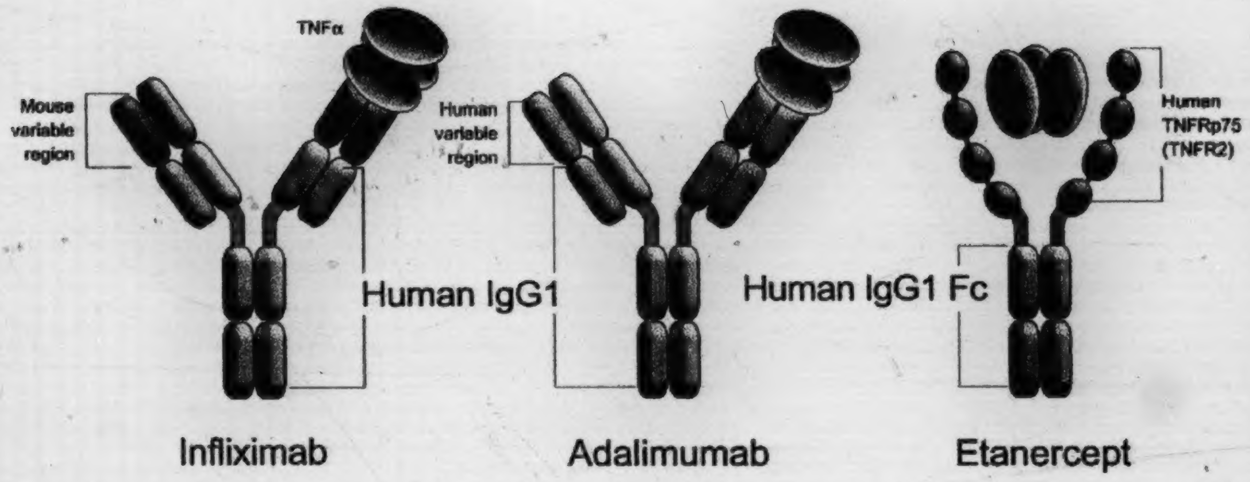

TNFα
Mouse variable region
Human IgG1
Human variable region
Human IgG1 Fc
Human TNFRp75 (TNFR2)
Infliximab
Adalimumab
Etanercept

Figure 1-5. Structure of TNFα binding drugs. Reproduced from Anderson PJ (2005) Tumor necrosis factor inhibitors: clinical implications of their different immunogenicity profiles. *Semin Arthritis Rheum* **34:**19-22.

juvenile RA. The first drug introduced was infliximab, a chimeric mouse-human anti-TNFα antibody. It effectively binds and inactivates soluble TNFα and causes complement and/or antibody dependent cell-mediated cytotoxicity to membrane bound TNFα (Markham and Lamb, 2000). Due to its murine constituent however, patients treated with infliximab can develop human anti-mouse antibodies to the drug. The incidence and significance of antibody development depends on patient factors such as concomitant infection and may be reduced by co-administration with methotrexate. The newest anti-TNF drug is adalimumab, a fully humanized anti-TNFα monoclonal antibody. Its mechanism of action is similar to infliximab but is associated with fewer incidence of antibody development due to its lack of animal components (Anderson, 2005). Etanercept is a soluble human recombinant tumor necrosis factor receptor comprised of the human p75 TNF receptor fused with Fc fragment of human IgG. Like adalimumab, auto-antibody development to etanercept is low (Anderson, 2005). Unlike the anti-TNF antibodies, etanercept binds primarily soluble TNFα as well as to lymphotoxin (also known as TNFβ) (Culy and Keating, 2002) and marked differences in action and pharmacokinetics between these three agents exist (Haraoui, 2005).

Because it is the most recently introduced drug, fewer studies are available for adalimumab, and most studies on conditions other than RA are with infliximab and etanercept. Although no studies directly compare the three drugs it appears that the drugs have different efficacy profiles. While all three agents are effective for the treatment of RA, some evidence indicates that patients who do not respond to etanercept or infliximab may benefit from switching to the other agent. It has been suggested that there exists a subset of the population who have lymphotoxin α driven disease rather than the more common TNFα dependent disease. For patients with TNFα dependent disease, response may be higher to infliximab due to more stable binding of infliximab to soluble and membrane bound TNF (Scallon, et al., 2002). For example, infliximab may be more effective than etanercept in Crohn's Disease due to the ability of infliximab but not etanercept to activate complement and induce apoptosis of activated T cells (Haraoui, 2005). Infliximab however, does not bind lymphotoxin whereas etanercept does. In a subset of adult RA patients and in all juvenile RA patients in whom lymphotoxin are detected, etanercept would theoretically be more effective. This is supported in results from clinical trials indicating that etanercept and not infliximab is effective for the treatment of juvenile RA (Haraoui, 2005).

Despite the well-established role of TNF in cardiovascular disease (Bruunsgaard, et al., 2000; Cesari, et al., 2003; Ridker, et al., 2000), therapy with TNF blocking agents has yielded mixed results. Anti-TNF therapy has been shown to improve endothelial dysfunction (Booth, et al., 2004; Hurlimann, et al., 2002) and was associated with a lower incidence of cardiovascular events in RA patients (Jacobsson, et al., 2005). Since TNFα may also have a pathogenic role in heart failure and is in fact produced by the failing myocardium, it was postulated that treatment with TNF blocking drugs would have therapeutic potential (Sarzi-Puttini, et al., 2005). Clinical trials have been conducted with infliximab and etanercept to determine whether the antagonism of TNF would be of benefit in patients with moderate to severe heart failure (Chung, et al., 2003; Mann, et al., 2004). Surprisingly, both studies demonstrated no benefit for patients treated with anti-TNF drugs and even an increase in death and hospitalizations for heart failure in patients treated with infliximab compared with patients given placebo. Several explanations have been suggested for these findings (Sarzi-Puttini, et al., 2005). At low, physiological concentrations, TNF may have a role in tissue remodeling and repair. It is possible that anti-TNFα therapies decrease TNFα concentrations to below physiological levels, thus worsening heart failure and increasing mortality. In addition to inactivating soluble TNF, infliximab may also have direct cytotoxic effects by binding membrane-bound TNF and fixing complement. In heart failure, this may have a deleterious effect, as myoctyes expressing TNF on its membranes may be targeted by infliximab resulting in complement mediated lysis. Etanercept acts as a carrier of soluble TNF, prolonging its half-life and increasing its concentration in serum (Culy and Keating, 2002). Although etanercept binds with high affinity, it also dissociates at a fast rate, therefore, high levels of TNF bound to etanercept remains immunoreactive which may result in the worsening of heart failure.

While the use of TNFα blocking agents should be avoided in patients with heart failure, the potential benefits of TNF antagonism on cardiovascular outcome in patients with RA should be examined further. Cardiovascular health in RA is influenced not only by a patient's inflammatory status, but also by a patient's response to treatment with cardiovascular drugs. It is not known if anti-TNF therapy influences the disposition and action of cardiovascular drugs known to be altered under inflammatory conditions.

Influence of Inflammation on Pharmacokinetics

Inflammation, in addition to its role in the pathogenesis of cardiovascular disease, also alters the pharmacokinetics of cardiovascular drugs that are efficiently cleared by the liver. Elevated plasma concentrations of a variety of drugs including propranolol, oxprenolol, and verapamil are observed in patients with inflammatory conditions such as Crohn's disease, celiac disease and rheumatoid arthritis (Kendall, et al., 1979; Mayo, et al., 2000; Schneider, et al., 1976). Initial evidence indicated that increased drug concentrations were related to markers of inflammation such as erythrocyte sedimentation rate (Kendall, et al., 1979; Schneider, et al., 1976) and pro-inflammatory cytokine levels (Mayo, et al., 2000). The causes of increased drug concentration include increased drug binding to plasma proteins resulting in reduced drug free fraction, and altered drug metabolism due to inflammation–induced suppression of drug metabolizing enzymes.

The importance of protein binding on inflammation-induced changes in drug concentration was demonstrated by a comparison of β-blocking drugs with differing extents of protein binding and routes of metabolism. Schneider et al investigated the plasma concentrations of propranolol, oxprenolol and metoprolol in patients with inflammatory disease (Schneider, et al., 1981). As expected, plasma concentrations of propranolol and oxprenolol, both highly protein bound and extensively metabolized drugs, were significantly elevated by inflammation. In contrast, plasma concentrations of the extensively metabolized but poorly protein bound drug metoprolol, were relatively unchanged. These findings supported the theory that increased plasma concentrations of β-blockers under inflammatory conditions were due to increases in protein binding secondary to increased α_1-acid glycoprotein concentrations (AAG). Plasma concentrations of AAG, an acute phase protein, are greatly increased in inflammatory conditions (Belpaire, et al., 1982; Nakamura, et al., 1993). Basic drugs such as the β-blockers and verapamil are extensively bound to AAG, and hence, are increased in inflammatory conditions such as arthritis. To further elucidate the mechanisms of this apparent interaction, numerous animal studies have also examined the effects of inflammation on drug disposition. Bishop et al observed that propranolol plasma concentrations were also elevated in adjuvant-arthritic rats after oral and intravenous doses (Bishop, et al., 1981). This was later attributed to increased protein binding rather than to changes in hepatic metabolism or blood flow (Walker, et al., 1986).

The question of whether changes in protein binding were solely responsible for altered drug disposition was further explored with other drugs in various other models of inflammation. When propranolol, metoprolol and antipyrine plasma concentrations were examined in turpentine-induced inflammation in the rat, it appeared that increased protein binding alone could not account for apparent increases in drug concentration (Belpaire, et al., 1989). Since metoprolol and antipyrine are minimally bound to plasma proteins, increased concentrations of these drugs were explained by a reduction in hepatic metabolism. The influence of endotoxin induced inflammation on the stereoselective pharmacokinetics of propranolol, oxprenolol and verapamil were also studied in the rat (Laethem, et al., 1994). As expected endotoxin treatment resulted in significantly increased protein binding for all three AAG-bound drugs, secondary to increased plasma levels of AAG. This resulted in elevated plasma concentrations of both enantiomers for all drugs tested, and the increase was more pronounced for the enantiomer for which protein binding was higher. In addition, unbound AUC increased and intrinsic clearance decreased for propranolol, oxprenolol and verapamil in the inflamed animals suggesting that impaired metabolic processes were involved as well. In patients with RA, oral clearance of verapamil was significantly reduced as a result of increased protein binding as well as decreased intrinsic clearance (Figure 1-6) (Mayo, et al., 2000). In IFNα-2a induced inflammation in the rat, verapamil clearance was also significantly reduced, although plasma protein binding was unaltered (Sattari, et al., 2003). Increases in drug concentration for highly protein bound and extensively metabolized drugs such as verapamil and propranolol in AA rats may therefore be explained by both protein binding changes and reductions in metabolism. In contrast, the pharmacokinetics of sotalol, a drug that is minimally bound to plasma proteins and not hepatically metabolized, was unaffected by inflammation in the rat (Kulmatycki, et al., 2001). The pharmacokinetics of valsartan has also been shown recently to be unaltered in patients with RA (Daneshtalab, et al., 2004). Valsartan is only about 20 % metabolized in the liver, but is approximately 92 % bound to plasma albumin. Despite extensive protein binding, however, plasma concentrations of the drug were comparable in patients with active and controlled RA as well as with healthy control subjects.

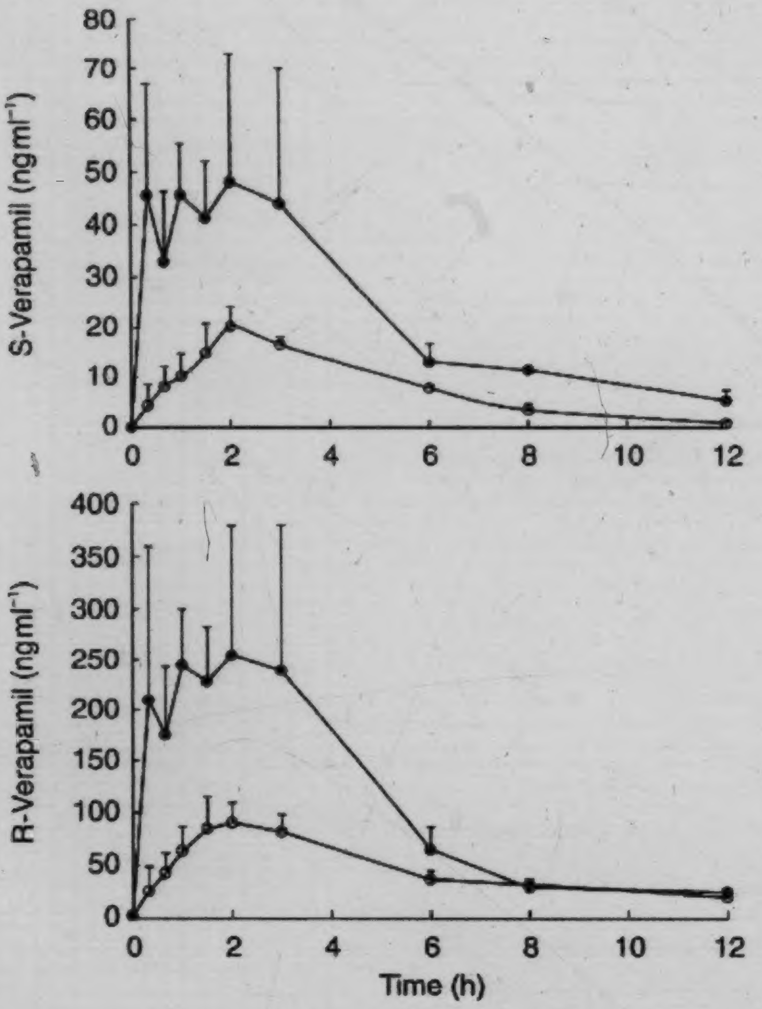


Figure 1-6. The effect of rheumatoid arthritis on serum verapamil concentrations. Open circles, healthy controls; solid circles, rheumatoid arthritis. Error bars represent the standard error of the mean. Reproduced from Mayo PR, Skeith K, Russell AS and Jamali F (2000) Decreased dromotropic response to verapamil despite pronounced increased drug concentration in rheumatoid arthritis. *Br J Clin Pharmacol* **50:**605-613.

Impaired drug metabolism is well established in inflammatory conditions in animals. Reduced cytochrome P450 content and microsomal oxidative enzyme activity was observed in rats following induction with adjuvant arthritis (Mortonand Chatfield, 1970). Pro-inflammatory cytokines such as IL-1β, IL-6, TNFα and IFNγ have been shown to decrease expression of various CYP isoenzymes and total P450 content in human hepatocyte cultures (Abdel-Razzak, et al., 1993; Muntane-Relat, et al., 1995). The mechanism by which cytokines alter cytochrome P450 content and activity may involve several pathways. Cytokines may downregulate CYP gene expression and protein directly at the transcriptional level (Abdel-Razzak, et al., 1993; Muntane-Relat, et al., 1995) or indirectly by stimulating the production of NO (Morgan, 1997). For example, IL-6 and TNFα inhibits the accumulation of CYP 1A1 and CYP3A4 mRNA and protein in human hepatocytes with no effect on protein decay cytokines (Muntane-Relat, et al., 1995). Nitric oxide may inhibit cytochrome P450 function directly by binding to the heme site (Khatsenko, et al., 1993) or indirectly through modulation of CYP mRNA and protein expression (Donato, et al., 1997; Khatsenko and Kikkawa, 1997). Other researchers have found down-regulation of cytochrome P450 to be independent of NO production as evidenced by the lack of effect of NO-donors on CYP gene and protein expression (Hodgson and Renton, 1995) and the inability of NOS inhibitors to reverse the effects of inflammation on CYP gene and protein expression (Sewer and Morgan, 1998). Others have noted that while cytokines inhibit the inducibility of CYP (1A and 3A) genes, the rate of mRNA translation and protein degradation was unaffected (Muntane-Relat, et al., 1995). It appears then that cytochrome P450 expression and activity are regulated by two complementary but independent mechanisms. Inhibition of CYP activity early after the onset of inflammation is likely the result of direct inactivation of CYP proteins whereas the continued suppression of CYP activity and protein expression may be due to cytokine and/or NO mediated reduction of CYP gene expression (Sewer and Morgan, 1998; Sewer, et al., 1998).

P-glycoprotein and Drug Transporters

P-glycoprotein (Pgp) is an ATP-dependent efflux protein that belongs to the large family of membrane associated drug transport proteins known as ATP-binding cassette transporters. Pgp was first identified for its role in conferring multi-drug resistance in tumor cells during chemotherapy and hence is also known as multi-drug resistant (MDR) protein. It is the product of the MDR 1 and 2 genes in humans and *mdr1a* and *1b* in mice. Efflux transporters are expressed on epithelial cells of many organs with excretory and/or metabolic functions such as the small and large intestine, liver, and kidney as well as at the blood brain barrier and placenta (Leslie, et al., 2005). In the gastrointestinal tract Pgp is localized at the brush border membrane of enterocytes, transporting substrates from within the cell back into the intestinal lumen. In hepatic and renal tissues, Pgp aids in the excretion of substrates into the bile and urine, respectively. On the apical membranes of brain and placental endothelium, Pgp protects the brain and fetus, respectively, from exposure to toxins and xenobiotics.

Pgp mediated efflux may have a significant impact on the oral absorption and bioavailability of some drugs. For example, the oral bioavailability of digoxin is significantly reduced in patients treated rifampin, a Pgp inducer (Greiner, et al., 1999). In addition, the AUC of digoxin after oral but not intravenous dosing is correlated with a 3.5 fold increase in intestinal Pgp content. Since renal clearance and half-life of digoxin are not altered by rifampin, it is evident that rifampin decreases digoxin bioavailability by reducing intestinal digoxin absorption via an increased Pgp mediated efflux of digoxin back into the gut lumen rather than through metabolic or renal clearance processes. Numerous other drug interactions involving altered drug transport have been documented. Decreased Pgp-mediated biliary clearance of digoxin (Angelin, et al., 1987; Angelin, et al., 1987) and doxorubicin (Booth, et al., 1998) by Pgp inhibitors have been reported as well as reduced hepatic uptake of cerivastatin by cyclosporin A inhibition of OATP1B1 (Shitara, et al., 2003). Conversely, induction of hepatic transport proteins on the canulicular membrane results in increased biliary clearance of substrates. In rats, tamoxifen, a Pgp substrate and inducer, increases its own biliary excretion and that of its metabolites along with increased expression of hepatic protein mdr1b mRNA (Riley, et al., 2000).

Many substrates for Pgp are also substrates for CYP3A enzymes. These include a wide range of structurally unrelated compounds but in general, tend to be large, hydrophobic, cationic compounds. Together with the CYP3A family of metabolic proteins, Pgps function largely as a mechanism to minimize exposure to xenobiotics and have been referred to as 'phase III' metabolism. Interestingly, induction of Pgp and CYP3A4 is regulated by a common pathway; transcriptional activation of the MDR1 gene or 3A4 gene via ligand binding to the nuclear factor PXR (Kullak-Ublick and Becker, 2003). The breast cancer drug paclitaxel for example is a substrate for Pgp and CYP3A4 and is as well, a ligand for PXR, thereby stimulating its own metabolism (Kullak-Ublick and Becker, 2003).

Pgp may alter drug exposure through modulating the expression of CYP enzymes by influencing the intracellular concentrations of substrates that induce or inhibit CYP enzymes (Schuetz, et al., 1996). For example, when the combination of vincristine, doxorubicin and dexamethasone is given for multiple myeloma, intracellular concentrations of vincristine and doxorubicin are decreased due to the combined effects of increased Pgp efflux of the drugs and increased metabolism by CYP3A due to the induction of both proteins by dexamethasone (Chandra and Brouwer, 2004). In contrast, Pgp inhibition by a substrate such as tacrolimus, may increase its own extent of metabolism through enhanced exposure to intracellular metabolism (Wu and Benet, 2003).

Efflux proteins form an important part of the blood brain barrier and has been the subject of recent reviews (Loscher and Potschka, 2005). Brain endothelial cells are characterized by tight junctions and supported by astrocytes that limit the exposure of the brain and cerebrospinal fluid to exogenous xenobiotics. Pgp is located at the luminal membrane of brain capilliary cells. The absence of Pgp in the blood brain barrier of *mdr1a* knockout mice results in the increase in brain penetration of Pgp substrates by up to 10 to 100 fold as can blockage of Pgp by Pgp inhibitors. The potent opioid loperamide for example is used for the treatment of diarrhea because of its low central nervous system (CNS) penetration and minimal CNS effects. Concurrent administration with the Pgp inhibitor quinidine, however, results in enhanced CNS effects of loperamide, including opiate-induced respiratory depression. Other drugs whose brain concentrations are influenced by Pgp transport include anti-cancer agents, immunosuppressants, steroid hormones, opioids, protease inhibitors, antidepressants, and anticonvulsants (Loscher and Potschka, 2005). Interestingly, Pgp is also found on the apical membrane of choroids plexus (Leslie, et al., 2005), where it may aid in the transport of drugs into cerebrospinal fluid, although the impact of this is unclear.

As blood enters the liver through the portal and hepatic vessels, movement of compounds into and out of hepatocytes is aided by several classes of membrane transporters. Uptake transport proteins aid in the transport of compounds across the sinusoidal membrane into the hepatocyte, while efflux proteins secrete drugs and their conjugated metabolites into the bile at the canulicular membrane. Several efflux proteins are also located at the sinusoidal membrane where they secrete drugs/metabolites from the hepatocyte back into systemic circulation.

At the basolateral or sinusoidal membrane are transporters such as the Na^+-taurocholate co-transporting polypeptide (NTCP) and the organic anion transport polypeptide (OATP) that aid in the hepatocellular uptake of organic anions such as bile salts and steroid conjugates as well as xenobiotics such as digoxin, fexofenadine, enalapril, pravastatin, and rifampin, among others (Chandra and Brouwer, 2004). It is not known how significant a role active transport plays in the disposition and hepatic clearance of these drugs. Also found at the hepatocyte basolateral membrane is the family of efflux proteins known as multidrug resistant associated proteins (MRP1)-3, 4, 5 and 6. The substrates for these proteins include daunorubicin, doxorubicin, etoposide, vincristine, acetaminophen glucuronide, azidothymidine and methotrexate. It is unclear at present what impact these proteins have on drug clearance, however, perfused liver studies have shown a selective transport of intracellularly formed metabolites back into systemic circulation (Milne, et al., 1997). For example, 80 and 90 % of intracellularly formed morphine glucuronide metabolites, morphine-3-glucuronide and morphine-6-glucuronide, respectively, were secreted back into venous outflow in the perfused liver compared with only 20 and 10 % secretion into bile.

Most efflux transporters are localized on the canulicular hepatocyte membrane and are responsible for the efflux of xenobiotics out of the hepatocyte and into the bile. Pgp is the most widely recognized efflux protein and is important in the hepatic extraction and biliary clearance of many drugs as well as for its role in enterohepatic re-circulation. Other transport proteins found at the canulicular membrane include the phospholipid transporter, MDR3, and bile salt pump, BSEP. The MRP2 protein is the only MRP found on the canalicular membrane and is important for the transport of phase-2 metabolic products such as bilirubin glucuronides, sulfated bile salts and glutathione conjugates out of the cell. MRP2 proteins also transport drugs such as cisplatinum, anthracyclins, vinca alkaloids, and methotrexate (Chandra and Brouwer, 2004).

Altered expression and function of efflux pumps in gene knockout animals and by drug and disease interactions yields varied responses depending on the specific substrates and metabolic processes involved. Studies in Pgp knockout mice have shown that for Pgp substrates tacrolimus, vinblastine and paclitaxel, absence of Pgp results in decreased drug clearance, despite unaffected biliary secretion (Chiou, et al., 2000). For other drugs such as cyclosporine, biliary secretion in Pgp knockout mice was actually increased perhaps due to induction of an efflux transporter. Lack of Pgp in knockout mice also did not significantly affect the disposition of Pgp substrates ketoconazole, morphine and dexamethasone (Schinkel, et al., 1995). These contradictory findings result from the complex relationship between protein transporters and the metabolism and clearance of the parent and metabolites.

Inflammation has been demonstrated to have varying effects on the expression and function of drug transporters (Fernandez, et al., 2004). Pro-inflammatory cytokines TNFα, IL-6, IL1β, and IFNγ have been shown to decrease gene and protein expression and function of intestinal Pgp in human colon carcinoma Caco2 cells. In animal models of inflammation, hepatic Pgp function and expression tend to be decreased by inflammation, however, in rat hepatocytes, IFNγ and TNFα causes increases in Pgp expression and function. Inflammatory mediators also tend to decrease Pgp function in the brain in animal models of inflammation. Other efflux proteins such as MRP2 in parenchymal hepatocytes (Nakamura, et al., 1999) have been shown to be down-regulated by pro-inflammatory cytokines TNFα, IL-1 and IL-6. In AA rats, hepatobiliary clearance of methotrexate, an MRP2 substrate (Masuda, et al., 1997), was significantly reduced, coinciding with reductions in MRP2 protein expression (Achira, et al., 2002).

Verapamil is often described as a prototypical substrate and inhibitor of Pgp. The disposition of verapamil, however, is negligibly influenced by Pgp as it has nearly 100% absorption from the gastrointestinal tract and shows little biliary excretion. It has been suggested that Pgp mediated efflux may play a role in intestinal metabolism of verapamil by increasing the exposure time of verapamil to enterocyte-based CYP metabolizing enzymes resulting in increased pre-hepatic metabolism (Johnson, et al., 2001). This observation however, was only demonstrated at very low drug concentrations. At the higher concentrations tested, which reflected physiological concentrations in the portal vein following oral dosing, Pgp appeared to be saturated. Another factor that was not addressed in this study was that of Pgp inhibition by verapamil. At higher concentrations, verapamil may have inhibited Pgp function. Inhibition of Pgp by verapamil has an important role in chemotherapy where the pharmacologically less active R enantiomer is administered as an adjunct to increase cellular uptake of cancer drugs such as doxorubicin and in studies of p-glycoprotein inhibition.

Influence of Inflammation on Pharmacodynamics

The influence of pro-inflammatory mediators on drug response has been recently reviewed (Kulmatycki and Jamali, 2005). Reduced pharmacotherapeutic response has been reported for various diseases in association with elevated inflammatory mediators. For instance, persistent TNF activation was associated with treatment failure in AIDS patients during highly active antiretroviral therapy (Aukrust, et al., 1999), and elevated TNFα was associated with induction of doxorubicin resistance in cancer (Kobayashi, et al., 1997), and insulin resistance in diabetes (Saghizadeh, et al., 1996). Elevated concentrations of IL-6 have also been linked to insulin resistance in cancer patients (Makino, et al., 1998), as well as to treatment failure in depression (Maes, et al., 1997a), and refractory schizophrenia (Lin, et al., 1998). The increased incidence of cardiovascular disease in the elderly may be due in part to the negative impact of inflammation on response to cardiovascular modifying drugs such as the L-type calcium channel blocker verapamil (Abernethy, et al., 1986; Abernethy, et al., 1993) and β-blockers (Anonymous 1992; Messerli, et al., 1998). Old age has been associated with increased concentrations of pro-inflammatory cytokines IL-1, IL-6, TNFα, sTNFRII, and CRP (Bruunsgaard, et al., 1999; Liao, et al., 1993). Increased IL-6 and CRP levels in the elderly predicted disability onset (Ferrucci, et al., 1999) and were associated with mortality (Harris, et al., 1999). Elevated levels of TNFα and CRP have also been linked to development of atherosclerosis and diabetes, respectively, in the elderly (Barzilay, et al., 2001; Bruunsgaard, et al., 2000). Obesity may be considered a chronic low level inflammatory state as reflected by elevated CRP (Visser, et al., 1999), TNFα (Dandona, et al., 1998) and IL-6 (Bastard, et al., 2000) concentrations that may induce insulin resistance and endothelial dysfunction associated with obesity and cardiovascular disease

(Yudkin, et al., 1999). Obesity and old age also have been associated with reduced responsiveness to verapamil (Abernethy and Schwartz, 1988).

Altered response to verapamil and propranolol has also been shown in patients with RA (Mayo, et al., 2000) and in experimental arthritis (Guirguis and Jamali, 2003), respectively. The reduced potency of these drugs is particularly striking since they occur in the presence of increased drug concentrations. Although high drug concentrations are normally expected to produce enhanced or toxic effects, elevated plasma levels of propranolol and verapamil by inflammation, results in reduced pharmacodynamic response (Figure 1-7) (Guirguis and Jamali, 2003; Mayo, et al., 2000; Sattari, et al., 2003). For these highly protein bound drugs, reduced potency may be attributed to the effects of increased protein binding, secondary to increased α_1-acid glycoprotein concentration, and/or altered function of L-type calcium channel and β-adrenergic receptors, respectively. In support of the latter concept, is the observation that pharmacodynamic response to sotalol is also reduced in inflammation (Kulmatycki, et al., 2001). Sotalol is a β-adrenergic and potassium channel blocker that is minimally bound to plasma proteins and undergoes little or no hepatic clearance. Hence, sotalol pharmacokinetics are not influenced by inflammation. These observations clearly suggest that inflammation has pathophysiologic effects on drug targets, i.e. receptors (β-adrenergic, Ca^{++} channel, and K^+ channel) that alter the drug concentration- effect relationship. What is a common feature among the above observations is the presence of pro-inflammatory mediators such as TNFα, IL-6 and nitrite. Pro-inflammatory

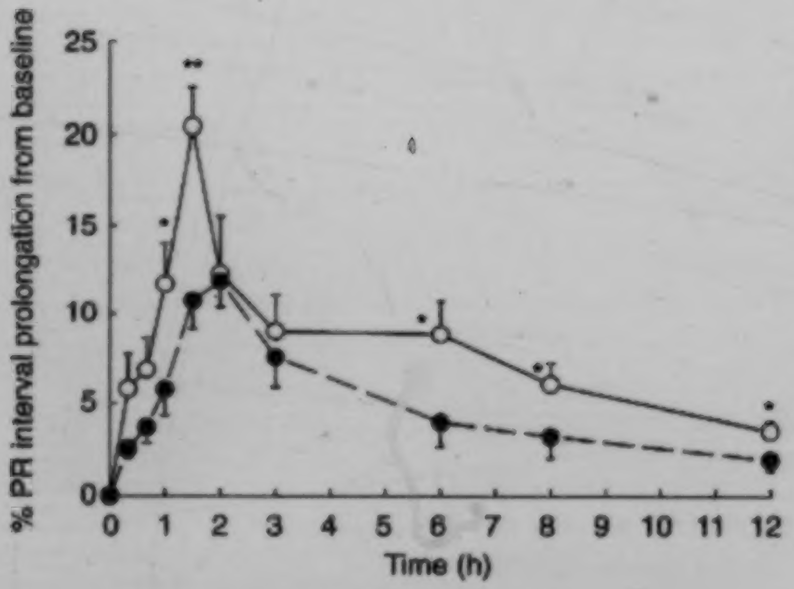


Figure 1-7. Reduced pharmacodynamic response to verapamil in rheumatoid arthritis. Open circles, healthy subjects; solid circles, rheumatoid arthritis. Error bars represent the standard error of the mean. Reproduced from Mayo PR, Skeith K, Russell AS and Jamali F (2000) Decreased dromotropic response to verapamil despite pronounced increased drug concentration in rheumatoid arthritis. *Br J Clin Pharmacol* 50:605-613.

mediators have been shown to down-regulate and/or inactivate receptors and channels (Kulmatycki and Jamali, 2001). For example, it has been demonstrated *in vitro* that binding of L-type calcium channels are lower in inflamed heart tissue (Sattari, et al., 2003). Importantly, *in vivo* administration of anti-TNFα monoclonal antibody reversed the suppressive effects of inflammation on pharmacodynamic response to sotalol along with a reduction in plasma TNFα andnitrite levels (Kulmatycki, et al., 2001). The effect that Kulmatycki et al observed, however, was not extensive, although similar observations have also been seen for atenolol and propranolol (Abouchehade, et al., 2001; Guirguis and Jamali, 2001).

Verapamil

Verapamil is a calcium channel blocker used in the treatment of hypertension, angina, and arrhythmia. In addition to symptomatic relief of angina and hypertension, newer calcium channel blockers have been shown to improve coronary endothelial function (ENCORE Investigators., 2003), slow the progression of asymptomatic carotid atherosclerosis (Zanchetti, et al., 2002), and reduce cardiovascular events in individuals with hypertension (Staessen, et al., 1997). Whether older agents such as verapamil exhibit these beneficial effects is less clear. Clinical trials have shown that verapamil can reduce mortality and morbidity in post-myocardial infarction patients without left ventricular dysfunction (Yusuf, et al., 1991) but was not better than diuretic or β-blocker therapy for preventing cardiovascular disease (Black, et al., 2003). Animal experiments have demonstrated that verapamil can suppress a number of calcium-dependent immune responses such as vascular cell adhesion molecule expression, endothelin release, platelet aggregation and iNOS expression (Bottadonna, et al., 1986; Haug, et al., 1998; Mustafa and Olson, 1999; Yamaguchi, et al., 1997). Verapamil mediated interruption of calcium dependent intracellular signaling therefore inhibits several inflammatory factors involved in atherosclerotic lesion development (Parmley, 1990).

Calcium signaling plays an essential role in many cellular processes. The calcium concentration gradient across cell membranes responsible for the contraction and relaxation of vascular smooth muscle cells is maintained by the L-type, voltage-sensitive calcium channel (Flynn and Pasko, 2000). L-type calcium channels are distributed throughout the body including the myocardium and vascular smooth muscle of the cardiovascular system and gastrointestinal tract. Currently, all calcium channel blocking drugs target the L-type voltage gated calcium channels, inhibiting calcium influx and activation of myosin fibres. The tissue selectivity of various calcium channel antagonists determines their clinical applications (Figure 1-8) (Nayler and Dillon, 1986). Verapamil acts on peripheral and myocardial smooth muscles to produce coronary and peripheral vasodilation. Verapamil also inhibits calcium influx through slow channels in the heart, thus exerting a direct negative inotropic effect (Nayler and Dillon, 1986). This energy sparing effect combined with coronary vasodilation is likely the reason for the usefulness of verapamil in treating angina. Verapamil also slows conduction

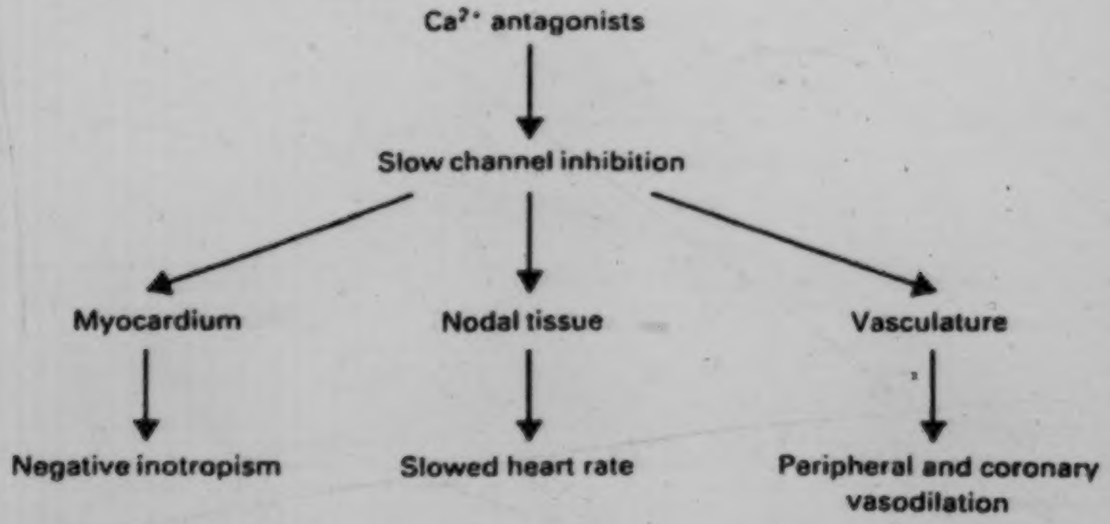

Ca²⁺ antagonists
Slow channel inhibition
Myocardium
Nodal tissue
Vasculature
Negative inotropism
Slowed heart rate
Peripheral and coronary vasodilation

Figure 1-8. The effect of calcium antagonism on the cardiovascular system. Adapted from Nayler WG and Dillon JS (1986) Calcium antagonists and their mode of action: an historical overview. *Br J Clin Pharmacol* **21 Suppl 2:**97S-107S.

through the atrio-ventricular node, increasing the effective refractory period, and is thus an effective anti-arrhythmic (Singh, 1986).

Verapamil is a highly permeable drug that is nearly completely absorbed from the intestinal tract after oral administration (Hoffman, et al., 1995; Johnston, et al., 1981). Oral bioavailability however, is low (~20%) due to extensive first pass hepatic metabolism. Verapamil is primarily metabolized by CYP3A4 and CYP1A2 to D-617 and norverapamil (Figure 1-9) (Kroemer, et al., 1993). Some O-demethylation of verapamil occurs via CYP2C enzymes, but the amounts *in vivo* are negligible (Busse, et al., 1995). Of the metabolites, only norverapamil is found in appreciable concentrations in plasma after oral dosing. Norverapamil concentrations can reach that of verapamil, but its pharmacological activity is only 20% of the parent drug. Verapamil exhibits extensive stereoselective binding to plasma proteins albumin and AAG. R-verapamil is approximately 92 to 94% bound to AAG and albumin compared with 86 to 88% binding of S-verapamil to the same proteins. As a result, plasma concentrations of the R enantiomer are significantly higher in plasma than the S enantiomer, although pharmacologic activity is attributed to S-verapamil and is related to plasma concentration (Figure 1-10). Notably, plasma protein binding selectivity is reversed in the rat, such that S-verapamil binds to a greater degree and has consequently higher plasma concentrations than R-verapamil. Peak plasma concentrations are reached about 1.5 hours after oral dosing and the mean elimination half-life ranges from 2.8 to 7.4 hours. Half-life ranges at steady 70state increase to 4.5 to 12 hours. Verapamil and its metabolites are eliminated in the urine; therefore dosage adjustments may be required in patients with hepatic and/or renal insufficiency.

1

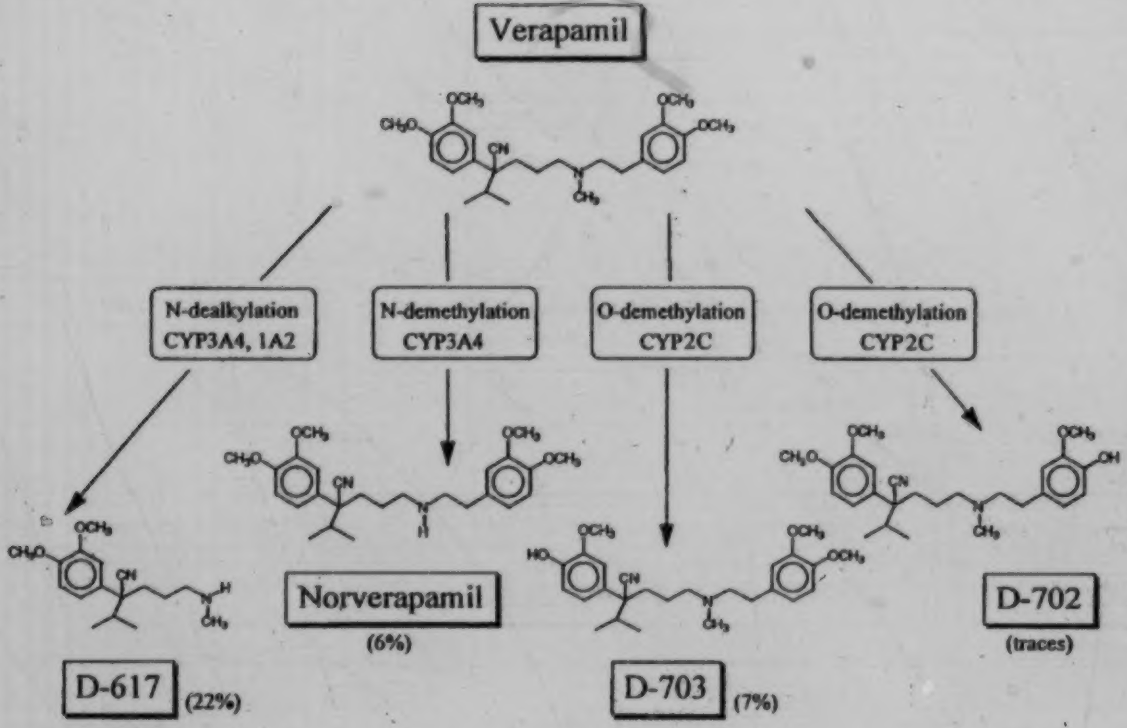

Verapamil
N-dealkylation
CYP3A4, 1A2
N-demethylation
CYP3A4
O-demethylation
CYP2C
O-demethylation
CYP2C
D-617
(22%)
Norverapamil
(6%)
D-703
(7%)
D-702
(traces)

Figure 1-9. Chemical structure of verapamil and its primary metabolites, major routes of in vivo oxidative metabolism and respective cytochrome P450 enzymes involved. Data in parentheses indicate the amount of metabolite excreted in urine. Adapted from Busse D, Cosme J, Beaune P, Kroemer HK and Eichelbaum M (1995) Cytochromes of the P450 2C subfamily are the major enzymes involved in the O-demethylation of verapamil in humans. *Naunyn Schmiedebergs Arch Pharmacol* **353**:116-121.

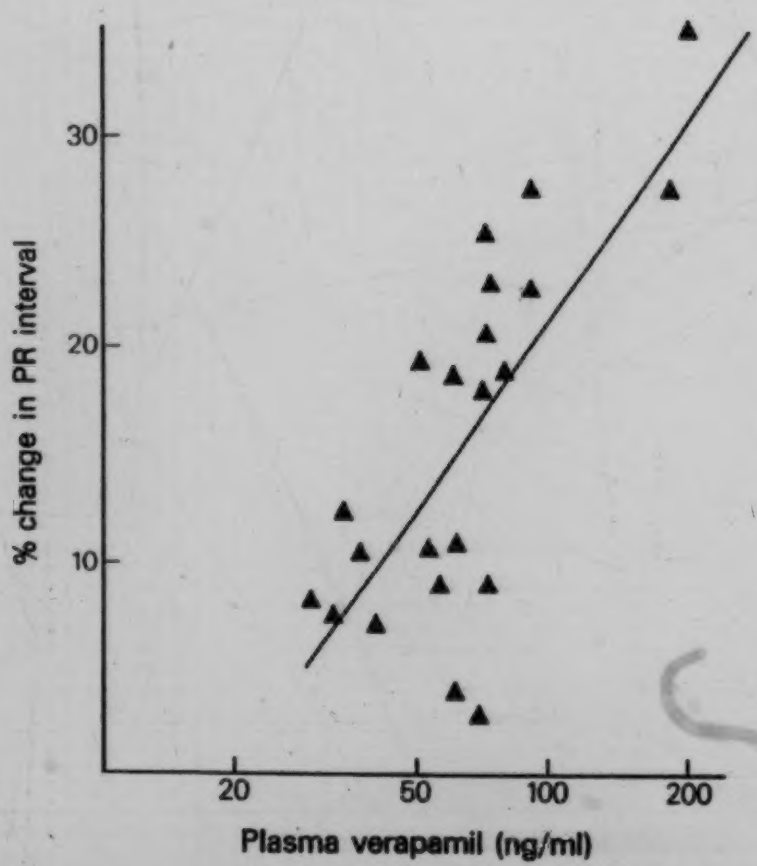


Figure 1-10. Percentage change in PR interval correlated with log plasma verapamil concentration after oral dose ($r = 0.732$, $P<0.001$). Adapted from Johnston A, Burgess CD and Hamer J (1981) Systemic availability of oral verapamil and effect on PR interval in man. *Br J Clin Pharmacol* **12**:397-400.

Study Rationale and Objectives

Animal Studies

The objectives of our animal studies were:

1. To determine whether cannulation surgery was of a nature and severity that would have lingering effects and thus affect subsequent stress responses.
2. To develop a model of chronic inflammation in the rat that would allow for the study of the effects of anti-TNF treatment on inflammation.
3. To investigate the effects of infliximab on verapamil metabolism and pharmacokinetics in the inflamed rat.

Animal models of disease are useful for conducting experiments in a controlled setting not easily carried out in human clinical studies. For pharmacokinetic studies, handling and surgical procedures add a component of stress that may influence pharmacokinetics of drugs perhaps through interaction with the immune system. Blood vessel catheterization is a common procedure in animal experimentation that facilitates the collection of biologic fluid samples while minimizing the stress due to handling. This surgical procedure however has been identified as a possible source of stress which may influence subsequent experimental procedures and response to stress. To assess whether lingering effects of stress would be a confounding factor in post-surgical rats following cannulation, we investigated the stress response in rats following cannulation surgery as a measure of recovery of animals to surgery by measuring the plasma corticosterone response to restraint stress using a newly developed, improved corticosterone HPLC assay.

Both acute and chronic models of inflammation have been shown to alter pharmacokinetics of efficiently cleared drugs. Acute models of inflammation such as those induced by lipopolysaccharide and IFNα-2a induce a rapid rise in inflammatory mediators, however, are self limiting and resolve spontaneously within days. To study the anti-inflammatory effects of anti-TNF drugs would require a model of inflammation that persisted for as long as the time needed for anti-TNF treatments to reverse the effects of inflammation. Adjuvant arthritis is a model of chronic inflammation that causes significant pain and suffering in experimental animals after the onset of disease, which typically occurs 2 to 3 weeks following adjuvant inoculation. There is sparse data to suggest that inflammatory markers begin to increase within days of adjuvant injection. We therefore endeavored to determine whether the early, asymptomatic phase of AA, pre-AA, would be an appropriate model of inflammation to study the effects of inflammation on verapamil pharmacokinetics and metabolism. Using pre-AA as an inflammatory model, we then studied the effects of infliximab on verapamil pharmacokinetics and metabolism.

Hypotheses

1. Cannulation surgery in the rat alters subsequent stress responses within the first week after surgery.
2. Pre-AA causes inflammation in the rat resulting in decreased verapamil metabolism and reduced verapamil clearance without causing undue pain and stress.

3. Treatment of pre-AA rats with infliximab decreases inflammatory markers and reverses the suppressive effects of pre-AA on verapamil metabolism and pharmacokinetics.

Clinical Investigation

The objective of the human study was to compare the effect of infliximab versus other anti-inflammatory therapy on verapamil pharmacokinetics and pharmacodynamics in patients with rheumatoid arthritis. Rheumatoid arthritis is associated with reduced cardiovascular response to verapamil and decreased clearance of the drug, due perhaps, to the effects of significant systemic inflammation. Infliximab is a biologic anti-rheumatic drug that reduces inflammation as well as slows disease progression. We conducted a clinical study in patients with RA who were considered in clinical remission induced either by infliximab or other anti-rheumatic treatment. Pharmacokinetic and pharmacodynamic response were measured and compared with healthy controls.

Hypothesis

1. Treatment with infliximab reverses the effects of systemic inflammation on pharmacokinetic and pharmacodynamic response to verapamil (i.e. reduced PR interval response and decreased verapamil clearance) compared with RA patients in remission due to other anti-rheumatic therapies and healthy controls.

CHAPTER 2

Effect of cannulation surgery and restraint stress on the plasma corticosterone concentration in the rat: application of an improved corticosterone HPLC assay.

Introduction

Blood vessel cannulation allows repeated blood sampling from conscious, unrestrained rats while avoiding the stress due to handling, restraint and anesthesia (Reilly, 1998; Suzuki, et al., 1997; Yoburn, et al., 1984). The process, and the presence of the catheter itself, however, potentially induces stress. Cannulation surgery and physiological changes that occur as a result of a chronic catheter may evoke a stress response as indicated by a rise in plasma corticosterone. Surgery may cause changes in hormonal and hemodynamic responses (Weissman, 1990) that are reduced when pre-surgery analgesics are given to decrease stress and pain (George, et al., 1974). Catheter infection, while uncommon in rats, is known to stimulate a stress response, and is a potential consequence of long-term catheterization (Bradfield, et al., 1992; Popp and Brennan, 1981). While an adequate recovery time from surgery (Fagin, et al., 1983) and aseptic surgical technique may minimize these effects, the precise time for full recovery is uncertain. Fagin et al. (1983), showed that while plasma corticosterone levels stabilized three to four days after cannulation, they remained elevated compared with uncannulated rats. This is not surprising since recovery of the hypothalamic-pituitary-adrenal axis to acute stress may be prolonged following a severe stressor (Fleshner, et al., 1995; Garcia, et al., 2000). An adequate recovery time is therefore required to minimize the effects of surgery on a subsequent stressor (Fagin, et al., 1983; Garcia, et al., 2000).

It would be advantageous to use a cannula within days of insertion. Besides the risk of infection in a chronic catheter, and the challenge of keeping the cannula patent for more than a week (Yoburn, et al., 1984), other physiologic changes (e.g. altered levels of plasma proteins) have been observed (Terao and Shen, 1983). While a 7-day recovery period from surgery allowed rats to respond to experimentally-induced stress (Fagin, et al., 1983), it is unclear whether or not a robust stress response could be detected 1 or 2 days following cannulation. To assess the optimum time to recovery from the stress of cannulation surgery, we studied the time-course of plasma corticosterone during recovery of the hypothalamic-pituitary-adrenal axis from jugular vein cannulation and the subsequent stress response.

High performance liquid chromatography (HPLC) methods for corticosterone analysis have been developed that are specific and sensitive (Wong, et al., 1994; Woodward and Emery, 1987). They, however, require long extraction times with large volumes of solvent. We report the development of a reversed-phase HPLC method with a simple extraction method for the determination of corticosterone in rat plasma.

Materials and Methods

Chemicals

Corticosterone and betamethasone were obtained from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) and ethyl acetate (reagent grade) were obtained from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Decolorizing charcoal (Norit A) was obtained from BDH Inc. (Toronto, ON, Canada).

Apparatus and chromatographic conditions

The HPLC apparatus consisted of a Model 590 pump, a 712 WISP autosampler (Waters, Mississauga, ON, Canada), a 10 cm x 4.6 mm I.D. C_{18} analytical column packed with 5-µm particles (Phenomenex, Torrance, CA, USA), and a HPLC pre-column insert packed with C_8 (Waters, MA, USA) particles. The detector was an SPD-10A ultraviolet visible detector (Shimadzu, Tokyo, Japan) set at 254 nm, and the recorder-integrator was a Model 3390A (Hewlett-Packard, Palo Alto, CA, USA). The mobile phase was acetonitrile-water-acetic acid-TEA (22:78:0.1:0.03, v/v), and was pumped at a flow-rate of 1.0 mL/min at ambient temperature.

Standard solutions

Stock solutions of corticosterone (2.5 µg/mL) and betamethasone (1.25 µg/mL) were prepared in ethyl acetate and stored at 4° C. Standard curves were prepared using pooled plasma treated with decolorizing charcoal to remove endogenous corticosterone (12,14,15). Decolorizing charcoal (0.05 g/mL of plasma) was added to rat plasma and stirred for 90 minutes at room temperature. The suspension was then centrifuged at 13 600 *g* for 8 minutes at 4-6° C. The plasma layer was decanted and filtered through a 0.45 μm Lida filter to remove carbon particles.

Sample preparation

To each 500 μL plasma sample, was added 50 μL of internal standard (betamethasone, 1.25 μg/mL). This mixture was extracted with 5.0 mL of ethyl acetate, vortex-mixed for 30 seconds, followed by centrifugation at 1800 *g* for 10 min. The organic layer was transferred to clean tubes, then washed with 1.0 mL of 0.1 *M* NaOH and then with 1.0 mL of water, and centrifuged for 5 minutes. The organic layer was transferred to clean tubes, and evaporated to dryness. The residue was reconstituted in 170 μL of mobile phase and 150 μL was injected into the HPLC system.

Extraction efficiency

To determine the extraction efficiency of corticosterone from plasma, 0.5 mL of plasma was spiked with corticosterone at concentrations of 10, 100, and 500 ng/mL and extracted as described above. Unextracted standards consisted of corticosterone solutions prepared at the same concentrations, evaporated to dryness, then reconstituted with mobile phase, and injected into the HPLC system. Percentage recovery was calculated as follows:

*Recovery (%) = (peak area of extracted plasma/peak area of unextracted standard)*100*

Accuracy and precision

Plasma samples spiked with corticosterone at concentrations of 10, 25, 50, 100, 250, and 500 ng/mL were analyzed according to the procedure described above. Precision was determined by the calculation of inter- and intra-day co-efficients of variation (% C.V.). Accuracy was calculated as percentage error: *% error = (observed concentration – expected concentration)/expected concentration *100*

Animal study

This study was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Male, Sprague-Dawley rats (n=24) weighing between 250 and 300 g (Health Sciences Laboratory Animal Services, University of Alberta, Edmonton, Canada) were housed in the study area 24 hours prior to cannulation surgery, with a 12 h light/dark cycle (lights on at 8:00 a.m.). Under pentobarbital sodium anesthesia (65 mg/kg i.p.; Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada), polyethylene (PE-50; Clay Adams, Parsippany, NY, USA) cannulas tipped with 2 cm of silastic (Dow Corning Corp, Midland, MI, USA) tubing, were inserted into the right jugular vein and exteriorized by subcutaneous tunneling to an incision made in the interscapular area. Rats were then allowed to recover overnight and had free access to food and water. Rats were divided into 4 groups and were exposed to stress on either 1, 2, 3 or 4 days (day 1, 2, 3, and 4) following surgery. On the day of experiment, 1.0 mL of blood was sampled from conscious rats immediately before (baseline) and after (stress) exposure to 1 hour of restraint stress to determine levels of corticosterone at baseline and in response to stress, respectively. Corticosterone levels of control (intact) rats (n=4) were measured by withdrawing blood from the tail-vein of intact rats under halothane/O_2 anesthesia. All blood samples were drawn between the hours of 8:00 and 11:00 a.m. After collection of blood samples, the plasma layer was separated by centrifugation and stored at –20° C until analysis.

Statistical Analysis

The significance of the observed differences between the control (intact) and baseline (cannulated) corticosterone was assessed using the one-way ANOVA followed by the Duncan's New Multiple Range test. For cannulated rats, the difference between baseline values and those after stress was tested using the paired Student's t-test. For all tests, differences were accepted as significant when α was at most 0.05.

Results

Corticosterone assay

Representative chromatograms of untreated plasma, charcoal-treated plasma, and charcoal-treated plasma spiked with internal standard and 100 ng/mL of corticosterone are shown in Figure 2-1. Endogenous corticosterone was present in untreated plasma, with a peak retention time of 22.3 minutes (Figure 2-1A). No interfering peaks were observed in the charcoal-treated plasma, at the retention time of corticosterone or internal standard (Figure 2-1B). The retention time was 17.3 minutes for the internal standard peak (Figure 2-1C), and 22.5 minutes for corticosterone (Figure 2-1D). Standard curves showed excellent linearity ($r > 0.99$) between the peak-area ratios of corticosterone:internal standard and corticosterone concentration. A typical standard curve could be described by the equation, $y = 0.0095x - 0.0022$ over a range of 10 – 500 ng/mL. As shown in Table 2-1, accuracy of the assay was within 7.9% of the spiked standards and the intra- and inter-day coefficient of variation (C.V.) of the assay ranged from 1.0 to 6.9% and 0.8 to 10%, respectively. Extraction efficiency was at least 84% at each of the concentrations as shown in Table 2-2.

Animal study

Plasma corticosterone measured daily before restraint stress (baseline) were not significantly different from one day to another and were similar to corticosterone levels in the control (intact) group (Figure 2-2). Exposure to restraint stress significantly elevated corticosterone concentrations compared to the pre-stress levels, except on day 2 (Figure 2-2).

TABLE 2-1. Intra- and inter-day variability of assay of corticosterone spiked in rat plasma.

Spiked Concentration (ng/ml)	Observed Concentration (mean, ng/ml)	C.V. (%)	Accuracy (% difference)
Intra-day, n=6			
10	11	6.9	7.9
25	24	6.6	-4.2
50	48	4.9	-3.9
100	94	2.8	-5.8
250	264	3.7	5.5
500	496	1.0	-0.8
Inter-day, n=4			
10	11	6.9	4.9
25	24	6.0	-3.4
50	49	3.9	-3.1
100	100	10.0	0.3
250	259	4.2	3.5
500	498	0.8	-0.3
Quality Control, n=3			
55	55	1.3	-0.2
450	441	0.4	-2.1

TABLE 2-2. Extraction efficiency of the assay for corticosterone spiked in rat plasma (n=3).

Concentration (ng/ml)	Recovery (mean)(%)	C.V. %
10	85	8.7
100	87	6.5
500	84	6.1

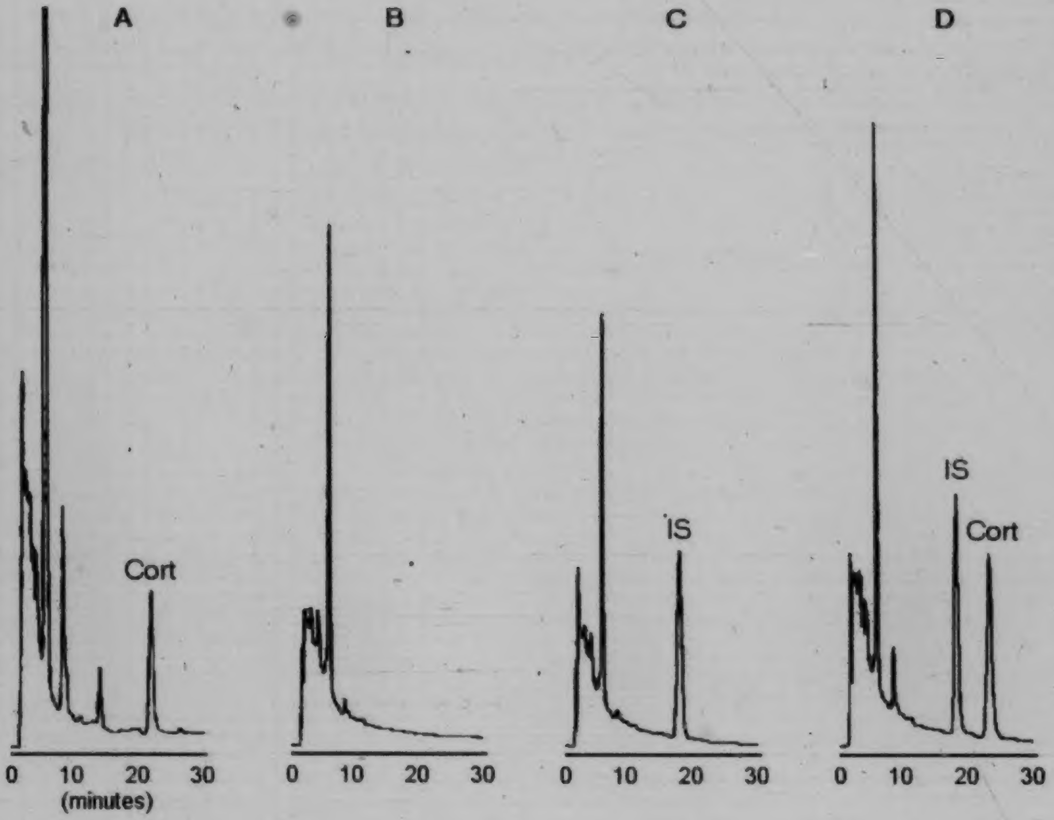


FIGURE 2-1. Chromatograms of untreated plasma containing endogenous corticosterone (Cort) (A), charcoal-treated plasma (B), charcoal-treated plasma spiked with internal standard (betamethasone) (C), and charcoal-treated plasma spiked with internal standard and corticosterone (100 ng/mL) (D).

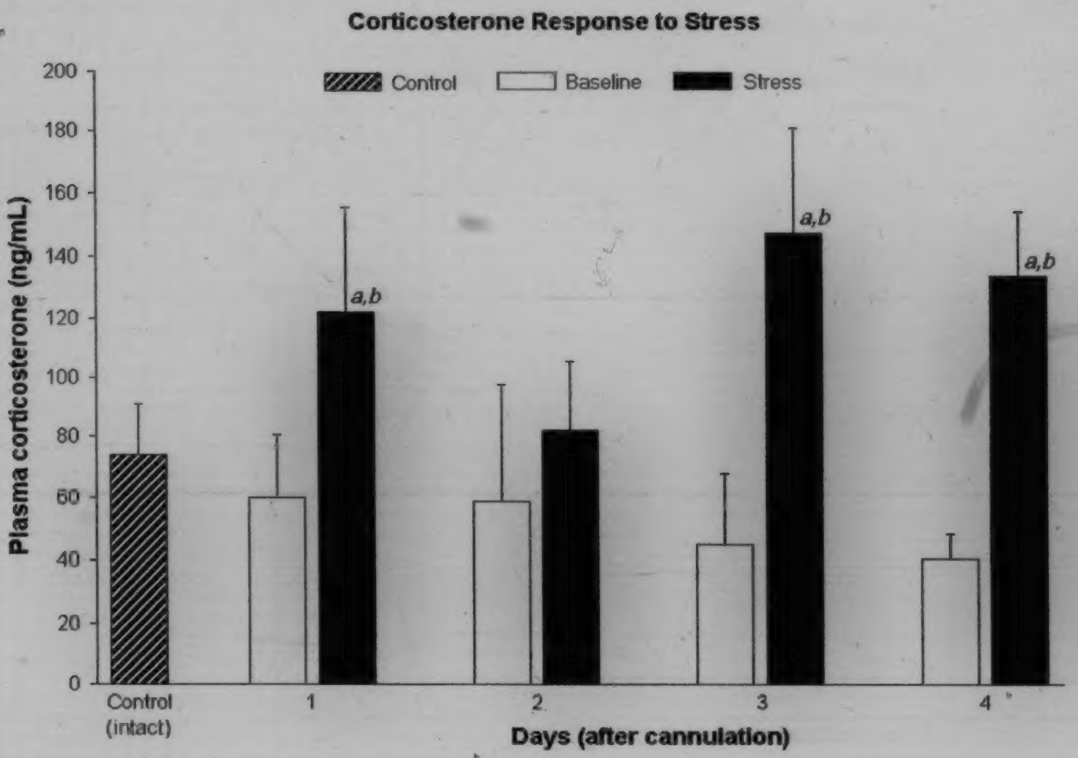


FIGURE 2-2. Plasma corticosterone in control (intact) rats and those before (baseline) and after 1 h restraint stress in cannulated rats. Data expressed as mean ± SD.

[a] Significantly different from control ($p<0.05$).

[b] Significantly different from baseline ($p<0.05$).

Discussion

We describe an HPLC method that is accurate and precise and suitable for determination of corticosterone in rat plasma. We used an established method, i.e., removing endogenous corticosterone by carbon treatment, for construction of our standard curves (Doppenschmitt, et al., 1995; Jusko, et al., 1994; Wong, et al., 1994). The extraction procedure was simple and rapid. A smaller volume of solvent, 5 mL ethyl acetate, was required, compared with previous assay (Wong, et al., 1994), which required 15 mL of methylene chloride. This extraction required only 30 seconds of vortex mixing compared with 15 minutes on a shaker (Wong, et al., 1994). This yielded good extraction efficiency, at least 84% at all concentrations, and very good accuracy and precision; accuracy within 7.9% of the spiked standards and the intra- and inter-day coefficients of variation of the assay ranged from 1.0 to 6.9% and 0.8 to 10%, respectively. The drug and internal standard were resolved with no interfering peaks. The peak elution times of 17 and 22 min were observed for internal standard and corticosterone, respectively which were longer than 9 and 12 min reported previously (Wong, et al., 1994). This is due likely to our use of a more polar mobile phase.

Our results show that baseline plasma corticosterone levels in jugular vein cannulated rats were not significantly different from basal corticosterone in uncannulated rats. These levels were similar to literature reports of corticosterone levels in jugular vein cannulated rats (Phelps, et al., 1995) and carotid artery cannulated rats after they had returned to baseline (Fagin, et al., 1983). Whereas Fagin *et al.* (1983), observed plasma corticosterone to be elevated initially after cannulation and then declining by day 3, we observed stable corticosterone levels for four days following surgery. This may be due to an inherent difference between jugular vein and carotid artery cannulation. Carotid cannulation is a more stressful procedure than jugular cannulation, and can cause marked weight loss as well as sympathetic nerve damage (Yoburn, et al., 1984).

Cannulated rats responded to 1 hour of restraint stress with elevated plasma corticosterone concentrations on all four days following surgery, although the rise was not significant on day 2. The lack of significance in corticosterone concentration on day 2 may be attributed to the high relative variability in the data on that day (Figure 2-2). Repeated blood sampling has been shown to increase plasma corticosterone levels, particularly in the first 0.5 h (Vachon and Moreau, 2001), and could possibly have contributed to the variability in our data. The effect of sampling time however, was likely minimal, as Vachon and Moreau (2001) demonstrated that the effect of sampling on corticosterone level is no longer apparent by 1 h following the first sampling time. Our post-stress blood samples were collected at least 1 h following the first blood sample. Furthermore, the rise in corticosterone levels in our study are a good reflection of the effects of restraint stress since peak corticosterone levels have been shown to occur at 1 h following 1 h of restraint stress (Garcia, et al., 2000).

In the case of a more intense stressor such as carotid artery cannulation, one would expect a less obvious rise in corticosterone level in response to stress due to the already elevated levels of corticosterone. In addition, it has been suggested that rats previously exposed to stress may exhibit a reduced response to a subsequent stressor due to a combination of negative feedback by corticosterone, and adaptation of the HPA axis (Marti, et al., 1999; Rivier and Vale, 1987). Our data however showed a robust corticosterone response to stress following surgery. This may be explained by evidence that the HPA axis is unlikely to show adaptation to a stressor that is different from the previous one (Armario, et al., 1988).

In summary, our data suggest that jugular vein cannulation does not alter subsequent stress responses and therefore may be used within days of the surgical procedure without the confounding effects of stress. The HPLC assay presented here is sensitive, reliable and simple. It has been used to assess plasma corticosterone levels in the rat following jugular vein cannulation, and subsequent response to stress.

CHAPTER 3

Effect of early phase adjuvant arthritis on hepatic CYP enzymes and pharmacokinetics of verapamil: an alternative approach to the use of an animal model of inflammation for pharmacokinetic studies.

Introduction

Adjuvant-induced arthritis (AA) is a well-established model of chronic inflammation in rats with symptoms of polyarthritis and inflammation developing typically 12 to 14 days post-adjuvant (Philippe, et al., 1997; Whitehouse, 1988). While this model of inflammation has been used extensively to study pharmacokinetics of drugs (Belpaire, et al., 1989; Emami, et al., 1998; Piquette-Miller and Jamali, 1993; Pollock, et al., 1989; Walker, et al., 1986), it subjects animals to significant pain (Nagakura, et al., 2003). AA causes increased expression of pro-inflammatory mediators which is associated with suppression of hepatic metabolic processes hence reduces clearance of otherwise efficiently cleared drugs (Kulmatycki and Jamali, 2001; Morgan, 1997; Piquette-Miller and Jamali, 1993). The elevated levels of pro-inflammatory mediators, however, appear to occur a few days after injection of adjuvants when animals are still asymptomatic and relatively free of the pain and distress associated with the disease progression (Coulthard, et al., 2002; Nagakura, et al., 2003; Philippe, et al., 1997; Szekanecz, et al., 2000). Since increased exposure to pro-inflammatory mediators may be responsible for inflammation-induced inhibition of drug clearance (Kulmatycki and Jamali, 2001), relevant pharmacokinetic data may be obtained during the early phase of AA and in the absence of the pain and stress associated with the experimental disease. We, therefore, evaluated the effect of the early phase of AA (pre-AA) on some pro-inflammatory mediator concentrations, hepatic CYP content, and po and iv pharmacokinetics of verapamil, a drug with well-known efficient hepatic metabolism (Laethem, et al., 1994; Mayo, et al., 2000; Sattari, et al., 2003). Since decreased clearance of drugs during inflammatory conditions may also be due to increased binding to plasma proteins secondary to an elevation of acute-phase protein α_1-acid glycoprotein (AAG) (Belpaire, et al., 1982), we also studied the effect of pre-AA on the protein binding of verapamil.

Materials and Methods

Chemicals

Verapamil hydrochloride, (+) glaucine, heptafluorobutanol, *Aspergillus* nitrate reductase (10 U mL^{-1}), HEPES, FAD, NADPH, lactic dehydrogenase (1500 U mL^{-1}), pyruvic acid, sulfanilamide, naphthylethylenediamine dihydrochloride, sucrose, potassium chloride, sodium chloride, bovine serum albumin, and Folin-Phenol reagent were purchased from Sigma-Aldrich (St. Louis, MO). Sodium dithionite and calcium chloride dihydrate were purchased from BDH (Toronto, ON, Canada). Copper sulfate, Tris (0.025 *M*)/Glycine (0.192 *M*) buffer, and Tris (0.025 *M*)/Glycine (0.192 *M*)/SDS (0.1%) buffer were purchased from MP Biomedicals (Irvine, CA). Sodium potassium tartarate, sodium bicarbonate, and Tween-20 were purchased from Fisher Scientific Co. (Pittsburgh, PA). Tris was purchased from Invitrogen (Carlsbad, CA). Ammonium persulfate, electrophoresis grade, was purchased from BioShop Canada Inc. (Burlington, ON, Canada). Sodium azide, *N,N,N',N'*-tetramethylethylenediamine, and 2-mercaptoethanol were purchased from EM Scientific (Gibbstown, NJ). Sodium dodecyl sulfate, Laemmli sample buffer and 40% acrylamide/Bis solution were purchased from Bio-Rad (Hercules, CA). High-performance liquid chromatography (HPLC) grade hexane and HPLC grade isopropanol, triethylamine, and 98% ethanol were purchased from Caledon Laboratories (Georgetown, ON, Canada). Heat-killed, dried *Mycobacterium butyricum* was purchased from Difco (Detroit, MI). Rat C-Reactive Protein ELISA kit was purchased from Helica Biosystems Inc. (Fullerton, CA). Rat TNFα Ultrasensitive ELISA kit was purchased from BioSource International (Camarillo, CA).

Animals

Experiments were performed on male, Sprague-Dawley rats (250-300 g), and were approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Animals had free access to water but food was withheld for 12 hours prior to pharmacokinetic experiments. They were housed under a 12 hour light/dark cycle.

Protocol

The day of adjuvant injection (0.2 mL of 50 mg/mL *Mycobacterium butyricum* suspended in squalene into the tail base) was marked as day 0. Control animals received 0.2 mL saline into the tail base. Animals were weighed, assessed for symptoms of arthritis including paw volume, nodule development and vocalization and blood samples were taken from the tail vein for the determination of serum nitrite, C-reactive protein and plasma TNFα concentrations on days 0, 1, 3, 5, and 6.

On day 5, the right jugular vein was cannulated for serial blood collection while animals were under halothane/O_2 anesthesia. Briefly, a polyethylene (PE-50; Clay Adams, Parsippany, NY) cannula tipped with 2 cm of silastic (Dow Corning Corp, Midland, MI) tubing was inserted into the right jugular vein and exteriorized by subcutaneous tunneling to an incision made in the interscapular area.

On day 6, pre-AA and control rats received single doses of racemic verapamil either intravenously (2 mg/kg in saline) (n=6/group) or orally via gastric gavage (20 mg/kg suspended in polyethylene glycol 400) (n=4-6/group). Blood samples (~0.2 mL) were collected at 0, 0.08, 0.17, 0.25, 0.5, 1, 2, 4 and 8 h after intravenous doses, and at 0, 0.25, 0.5, 0.75, 1, 2, and 4 h after oral doses. Plasma was separated and kept at -80° C for verapamil assay.

Microsomal Experiments

A separate set of control and pre-AA animals (n=4/group) were subjected to the protocol described above. They were sacrificed on day 6 and their livers removed and microsomes prepared (Barakat, et al., 2001). Briefly, livers were rinsed in 0.15 *M* KCl solution and homogenized in 0.25 *M* sucrose solution. The homogenates were centrifuged at 12000 *g* at 4° C for 10 min. The S9 fraction was collected and microsomes precipitated by addition of 1 *M* $CaCl_2$. The suspension was centrifuged at 27000 *g* at 4° C for 15 min, then the pellet was re-suspended in 0.15 *M* KCl solution and centrifuged at 27000 *g* at 4° C for 15 min. The pellet was again re-suspended in 0.25 *M* sucrose solution and stored at -80° C.

The microsomal protein concentration was determined by the Lowry method (Lowry, et al., 1951). Briefly, microsomes was incubated with 1% $CuSO_4$:2% Na.K.Tartarate:10% Na_2CO_3 anhydrous in 0.5 *M* NaOH (1:1:20 v/v/v) at room temperature for 10 min, then with 10% Folin-Phenol reagent at 50° C for 10 min. The sample was analyzed by spectrophotometry at 570 nm.

Total cytochrome P450 content was determined according to the method of Omura and Sato (Omura and Sato, 1964). Briefly, microsomes were suspended in 0.05 *M* phosphate buffer, pH 7.4, at a protein concentration of 1 mg/mL. A few milligrams of solid sodium dithionite were added to the 1 mL of suspension and a baseline determined using the recording spectrophotometer by scanning from 500 to 400 nm. Carbon monoxide was bubbled gently into the sample cuvette for 20 sec. The spectrum was again recorded from 500 to 400 nm. The quantity of cytochrome P450 was calculated from the optical density difference (450-480 nm) and the molar extinction coefficient of 91 $\text{m}M^{-1}\text{cm}^{-1}$.

Western Blot Analysis

CYP3A and CYP1A protein content was assessed by Western Blot analysis. Briefly, microsomal protein (30 μg) was denatured at 100° C for 5 min, then separated by SDS-polyacrylamide gel electrophoresis on 10 % polyacrylamide gels, and blotted onto a pure nitrocellulose membrane (0.45 μm, Trans-Blot® Transfer Medium, BioRad Laboratories, Hercules, CA). Nonspecific binding sites were blocked overnight in a solution of 5% skim milk:2% BSA:0.05% Tween-20 in TBS buffer. Membranes were incubated with primary antibody [1:1000 dilution; polyclonal rabbit anti-rat CYP3A2 or polyclonal goat anti-rat CYP1A1 (Daiichi Pure Chemicals, Tokyo, Japan)] for 2 h, and then with secondary antibody [1:5000 dilution; horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody or HRP-rabbit anti-goat IgG antibody, (Jackson Immunoresearch Laboratories Inc., West Grove, PA)] for 1 h. Immunoreactive proteins were visualized by chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences, England) and band density measured by densitometry (TBX Densitometer, Tobias Associates Inc., Ivyland, PA).

Verapamil Protein Binding

Protein binding was measured in serum obtained from the set of rats used for microsomal experiments. One mL serum from pre-AA (n=4) and control (n=4) rats were adjusted to pH 7.4 with 0.1 N HCl. The serum was spiked with 2000 ng/mL of racemic verapamil to approximate maximal serum concentrations of verapamil in rats after iv dosing. The serum was incubated at 37° C for 1 h, then transferred to Amicon® micropartition chambers (Amicon Corporation, Bedford, MA) for ultrafiltration at 2000 *g* for 1 h. Filtrate and nonfiltrate verapamil concentrations were measured by HPLC. The fraction unbound, f_u, was determined as, $f_u = C_u/C_t$, where C_u is the unbound concentration and C_t is the total concentration.

Stereospecific Verapamil assay

A stereospecific high performance liquid chromatography (HPLC) method (Shibukawa and Wainer, 1992) was used to determine plasma concentrations of R- and S-verapamil. Briefly, 75 µl of (+)-glaucine (400 ng/mL) as internal standard was added to 100 µl plasma, followed by 100 µl of 2 *M* NaOH and 0.4 mL sodium phosphate buffer (pH 7.0, ionic strength 0.1). Verapamil was extracted with 6 mL heptane:heptafluorobutanol (99:1) and vortex mixed, followed by centrifugation. The organic layer was evaporated to dryness, the residue reconstituted in mobile phase (hexane-isopropanol-ethanol-TEA, 92:4:4:0.1v/v/v/v) and injected into an isocratic HPLC system at a flow rate of 0.7 mL/min. The HPLC apparatus consisted of a Waters WISP 712 autoinjector (Milipore-Waters, Mississauga, Canada), an achiral column (5 cm x 4.6 mm I.D. Supelcosil LCSi, Supelco Inc., Bellefonte, PA) and chiral column (250 mm x 4.6 mm I.D., 5 µm Chiralpak AD-H column, Daicel Chemical Inc., Tokyo, Japan) maintained at 31°C, a 474 fluorescence detector (Waters, Mississauga, Canada) set at excitation of 272 nm and emission at 317 nm with a bandwidth at 18 nm, and a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA). Standard curves were linear over the concentration range of 10 – 1000 ng/mL ($r^2 \geq 0.99$, cv $\leq 10\%$). The minimum quantifiable concentration was 5 ng/mL for S- and R-verapamil.

Serum Nitrite Analysis

Serum nitrite (NO_2^-), a stable breakdown product of nitric oxide (NO), was measured using a previously described method (Grisham, et al., 1996). Briefly, nitrate (NO_3^-) was reduced to nitrite (NO_2^-) by incubating 100 µl of serum with 10 µl of *Aspergillus* nitrate reductase (10 U/mL) in the presence of 25 µl 1 *M* HEPES (pH 7.4), 25 µl 0.1 m*M* FAD and 50 µl 1 m*M* NADPH for 30 min at 37° C. Then, 5 µl of lactate dehydrogenase (1500 U/mL) and 50 µl of 100 m*M* pyruvic acid were added and incubated for an additional 10 min at 37° C. NO_2^- was determined by addition of 1.0 mL Griess reagent and absorbance measured at 543 nm. Standard curves were linear over the concentration range of 3 – 200 µ*M* ($r^2 \geq 0.99$, cv $\leq 10\%$). The minimum quantifiable concentration was 3 µ*M*.

Serum C-reactive Protein Analysis

A commercially available rat-CRP ELISA kit (Helica Biosystems, Inc., Fullerton, CA) was used. This assay required 100 µl of serum (1:10 000 dilution) to be added to a 96 well plate coated with antibodies to rat-CRP. After incubation for 30 min, the plate was washed and 100 µl of Conjugate (horseradish peroxidase (HRP)-labeled rabbit anti-rat CRP-IgG) was added and incubated for 30 min. The plate was again washed and 100 µl of TMB substrate solution was added and incubated for 10 min. Stop Solution (100 µl) was added to stop the reaction and absorbance was read at 450 nm. Standard curves were linear over the concentration range of 17.5 – 133 µg/mL ($r^2 \geq 0.99$, cv ≤10%). The limit of detection of the assay was 2.5 µg/mL.

Plasma TNFα Analysis

Plasma TNFα concentrations were measured using a commercially available rat-TNFα ultrasensitive ELISA kit (Biosource International, Camarillo, CA). Briefly, 100 µl of plasma was added to a 96 well plate coated with anti-rat-TNFα capture antibody. After incubation for 3 hours, the plate was washed and 100 µl of Biotin Conjugate added and incubated for 45 minutes. The plate was again washed and 100 µl of Streptavidin-HRP added and incubated for 45 minutes. After washing, 100 µl of Chromogen was added and incubated for 20 minutes followed by addition of 100 µl of Stop Solution and the absorbance read at 450 nm. Standard curves were linear over the concentration range of 2.3 - 150 pg/mL ($r^2 \geq 0.99$, cv ≤10%). The limit of detection of the assay was 1.9 pg/mL.

Data Analysis and Statistics

Pharmacokinetic indices for S and R verapamil after iv administration were estimated using WinNonlin 4.1 (Pharsight, Mountain View, CA). The open two-compartment model best described the unweighted data based on the Akaike information criteria. Pharmacokinetic indices for S and R verapamil after oral administration were determined by noncompartmental analysis. Elimination rate constants (β) were calculated using log-linear regression of at least three points in the log-linear terminal phase of the plasma concentration-time curve. The area under the plasma concentration-time curve (AUC) was calculated using the log-linear trapezoidal rule from 0 h to the time of the last measured plasma concentration (C_{last}). Extrapolation to infinity ($AUC_{t-\infty}$) was determined by C_{last}/β, and $AUC_{0-\infty}$ was determined as the sum of AUC_{0-t} and $AUC_{t-\infty}$. The ratios of oral to iv AUC were estimated by comparing the partial (0-4 h) AUCs after oral and iv doses.

Data are presented as mean ± standard deviation. Significance of difference in pharmacokinetic parameters between pre-AA and control groups were determined by the two-tailed Student's t-test at α=0.05. Comparison of animal weight, serum nitrite and CRP concentrations between the groups and between the experimental days were done by analysis of variance for repeated measures with Tukey's adjustment for multiple comparisons.

Results

Pre-AA rats gained significantly less weight than control rats (day 6: 307 ± 30 g vs 358 ± 26 g, p=0.001). They, however did not develop arthritic symptoms, i.e. signs of joint swelling, deformity and immobility.

Serum nitrite concentrations were significantly elevated in pre-AA rats by day 3 compared with control rats and remained elevated through day 6 (Figure 3-1). C-reactive protein concentrations were also significantly elevated in pre-AA rats as early as 1 day post-induction compared with control rats and remained elevated through day 6 (Figure 3-1). TNFα concentrations were elevated slightly but significantly only on day 6 (14.4 ± 4.7 pg/mL vs 9.2 ± 1.8 pg/mL, $p=0.015$) in pre-AA rats.

Pre-AA significantly increased plasma concentrations of verapamil and altered pharmacokinetic indices following both iv and oral administration (Figures 3-2 and 3-3). Following iv administration, $AUC_{0-\infty}$ increased by 165% for S-verapamil and 45% for R-verapamil (Table 3-1). A corresponding decrease in clearance was observed (58% S-verapamil; 29% R-verapamil). The apparent volume of distribution at steady state (Vss) and volume of the central compartment (Vc) were unchanged by pre-AA. The unbound fraction of drug was also significantly reduced by 80% for S and 67% for R-verapamil compared with controls.

Verapamil administered orally to pre-AA rats resulted in significantly elevated plasma concentrations of both enantiomers as compared with control rats and to a greater extent than after iv doses. Cmax and AUC_{0-4h} for S-verapamil in pre-AA rats were 6.7 and 9.3-fold higher than in control rats (Table 3-1). For R-verapamil Cmax and AUC_{0-4} in pre-AA rats were 3.6 and 9.5-fold higher than in control rats. The last measurement was made at 4 h, at which time the elimination phase had not been reached in pre-AA rats. Therefore, estimates of oral clearance and volume of distribution could not be determined. Absolute oral bioavailability also could not be obtained, however, the relative ratio of AUCs after oral dosing for the four hours post-dose was estimated (Table 3-1). The AUCpo/AUCiv ratio increased 3.7 fold for S-verapamil and 6.5 fold for R-verapamil pre-AA rats.

Total microsomal cytochrome P450 content in pre-AA rats were significantly reduced (49%) compared with control rats (p=0.001) (Figure 3-4). The reduction in total P450 content was significantly correlated with increases in serum pro-inflammatory mediators concentrations (Figure 3-5).

Microsomal content of CYP3A1/2 and CYP1A1/2 isoforms were also significantly down-regulated in pre-AA rats (Figure 3-4) and the reduction was correlated with the reduction in total P450 (Figure 3-5). The suppression of both CYP3A1/2 and CYP1A1/2 protein expression was also significantly correlated with changes in serum nitrite and TNFα concentrations, but not CRP levels (Figure 3-6).

TABLE 3-1. Pharmacokinetic indices of verapamil in control and pre-AA rats following intravenous 2 mg/kg (n=6/group) and oral 20 mg/kg administration (n=4-6/group).

	Control	Pre-AA
i.v.[a]		
A (ng/mL)		
S	1601 ± 1140	5961 ± 10103
R	1206 ± 678	2203 ± 3065
α (1/h)		
S	8.05 ± 4.56	7.54 ± 7.83
R	8.60 ± 4.28	7.53 ± 7.58
B (ng/mL)		
S	252 ± 114	589 ± 488
R	226 ± 92	251 ± 186
β (1/h)		
S	0.81 ± 0.28	0.69 ± 0.27
R	0.64 ± 0.19	0.51 ± 0.26
$AUC_{0-\infty}$ (μg.min/mL)		
S	30.3 ± 2.7	79.6 ± 38.5*
R	29.8 ± 2.1	42.9 ± 9.2**
CL (L/min/kg)		
S	0.033 ± 0.003	0.015 ± 0.006***
R	0.034 ± 0.002	0.024 ± 0.005***
Vc (L/kg)		
S	1.1 ± 0.4	0.6 ± 0.6
R	1.6 ± 0.3	1.5 ± 0.8
Vss (L/kg)		
S	1.8 ± 0.7	1.1 ± 1.0
R	2.5 ± 0.7	2.5 ± 1.3
f_u		
S	0.091 ± 0.051	0.019 ± 0.017**
R	0.118 ± 0.063	0.039 ± 0.019*
p.o.[b]		
C_{max} (ng/mL)		
S	173 ± 47	1057 ± 817*
R	37 ± 11	132 ± 63**
AUC_{0-4} (μg.min/mL)		
S	26.3 ± 8.4	245.6 ± 218.9*
R	4.7 ± 1.0	44.7 ± 48.5
$AUC_{(0-4h)}$ ratio (po/iv)		
S	0.10	0.37
R	0.02	0.13

A, distribution intercept; α, distribution rate constant; B, elimination intercept; β, elimination rate constant.

*p<0.05, **p<0.01, ***p<0.001, significantly different from control.

[a] Estimated by two-compartment analysis.

[b] Calculated by non-compartmental analysis.

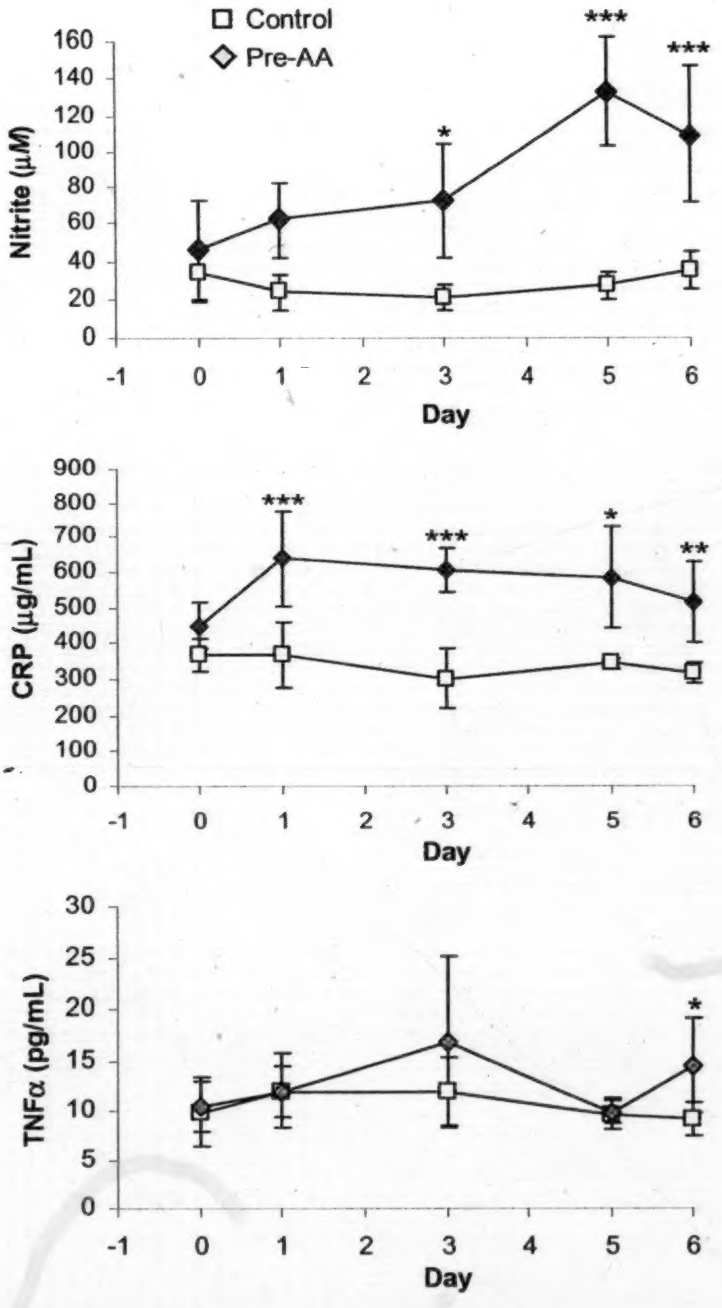


FIGURE 3-1. Mean serum nitrite, C-reactive protein and plasma TNFα concentrations in control (n=3-7) and pre-AA (n=3-9) rats. Error bars represent standard deviation of the means. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from control.

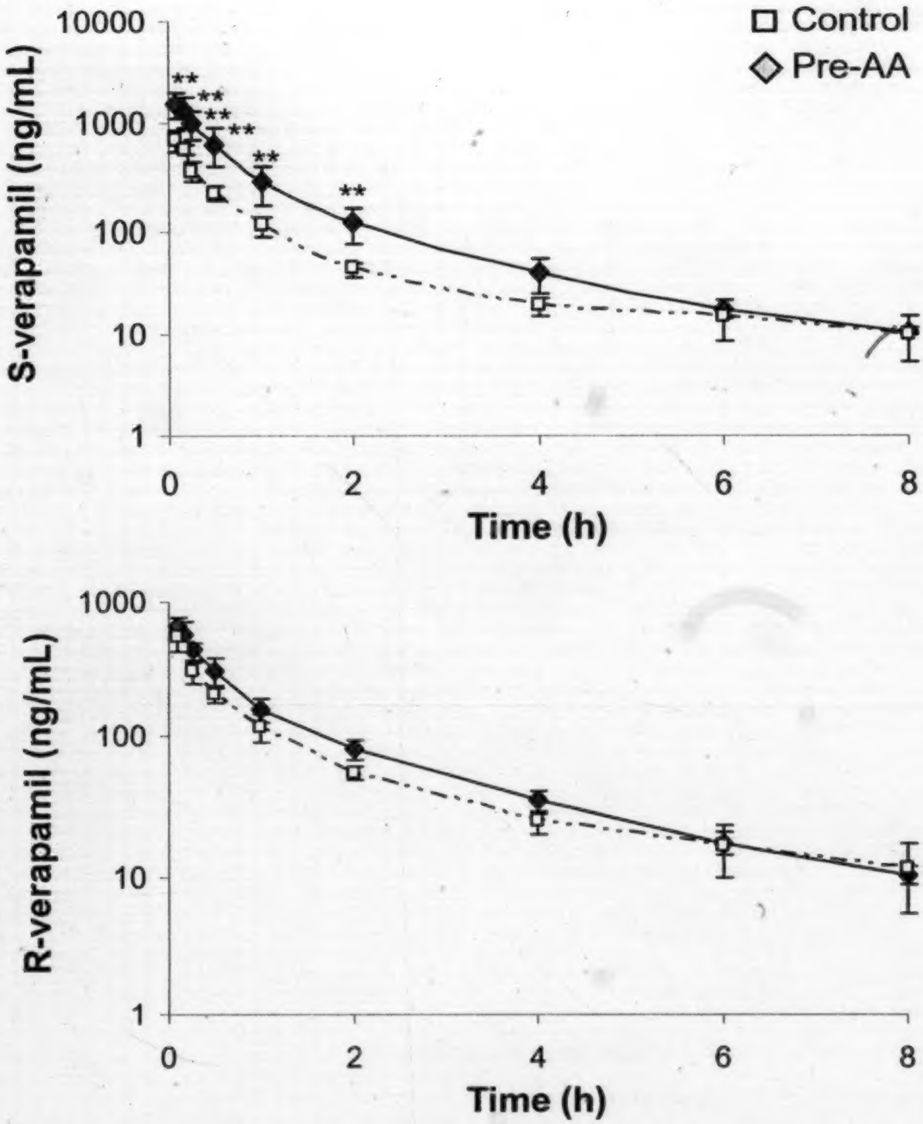


FIGURE 3-2. Mean plasma verapamil concentration versus time curves of control (n=6) and pre-AA (n=6) rats following administration of single intravenous 2 mg/kg racemic verapamil doses. Error bars represent standard deviation of the means. Lines connect experimental data points. **p<0.01, significantly different from control.

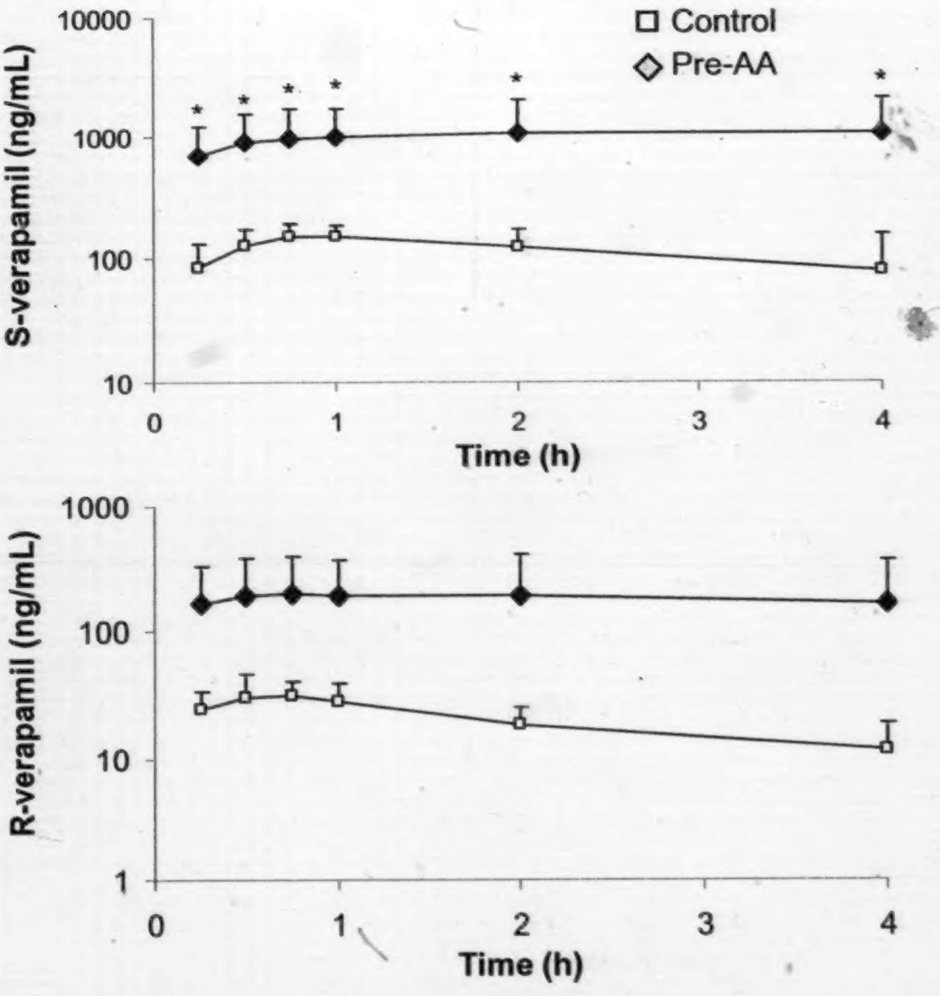


FIGURE 3-3. Mean plasma verapamil concentrations of control (n=6) and pre-AA (n=4) rats following administration of single oral 20 mg/kg racemic verapamil doses. Error bars represent standard deviation of the means. Lines connect experimental data points. *$p<0.05$, significantly different from control.

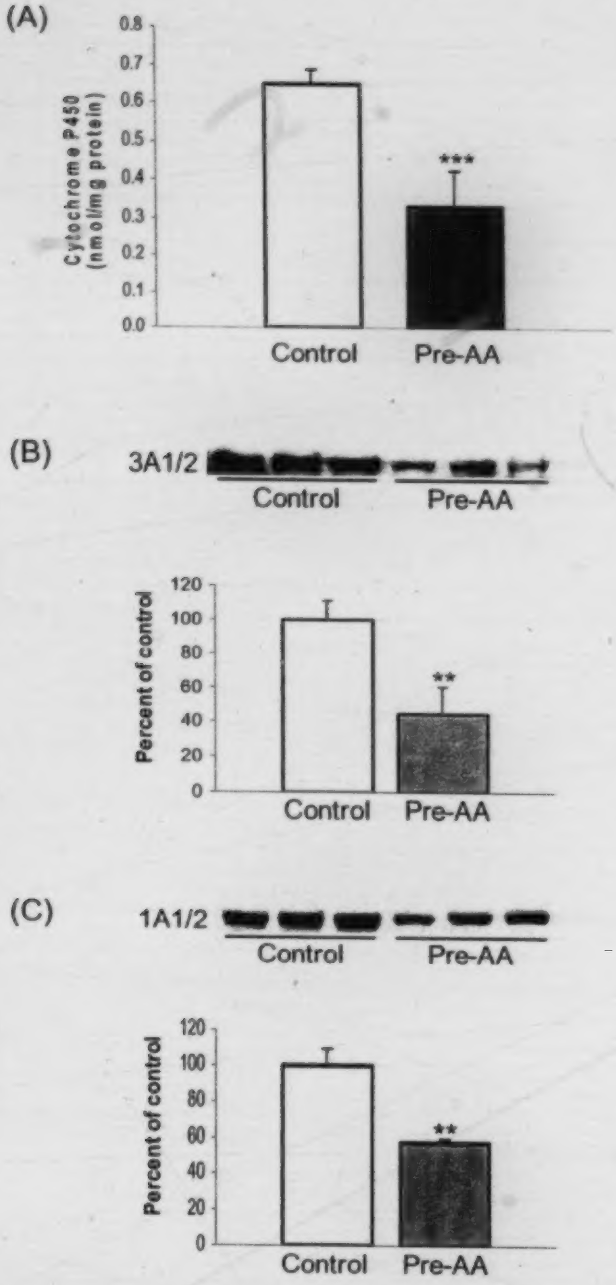


FIGURE 3-4. (A) Mean total cytochrome P450 content in control (n=4) and pre-AA (n=4) rats; (B) CYP3A1/2 western blot and protein content in control (n=3) and pre-AA (n=3) rats; (C) CYP1A1/2 western blot and protein content in control (n=3) and pre-AA (n=3) rats. Error bars represent standard deviation of the means. $^{**}p<0.01$, $^{***}p<0.001$, significantly different from control.

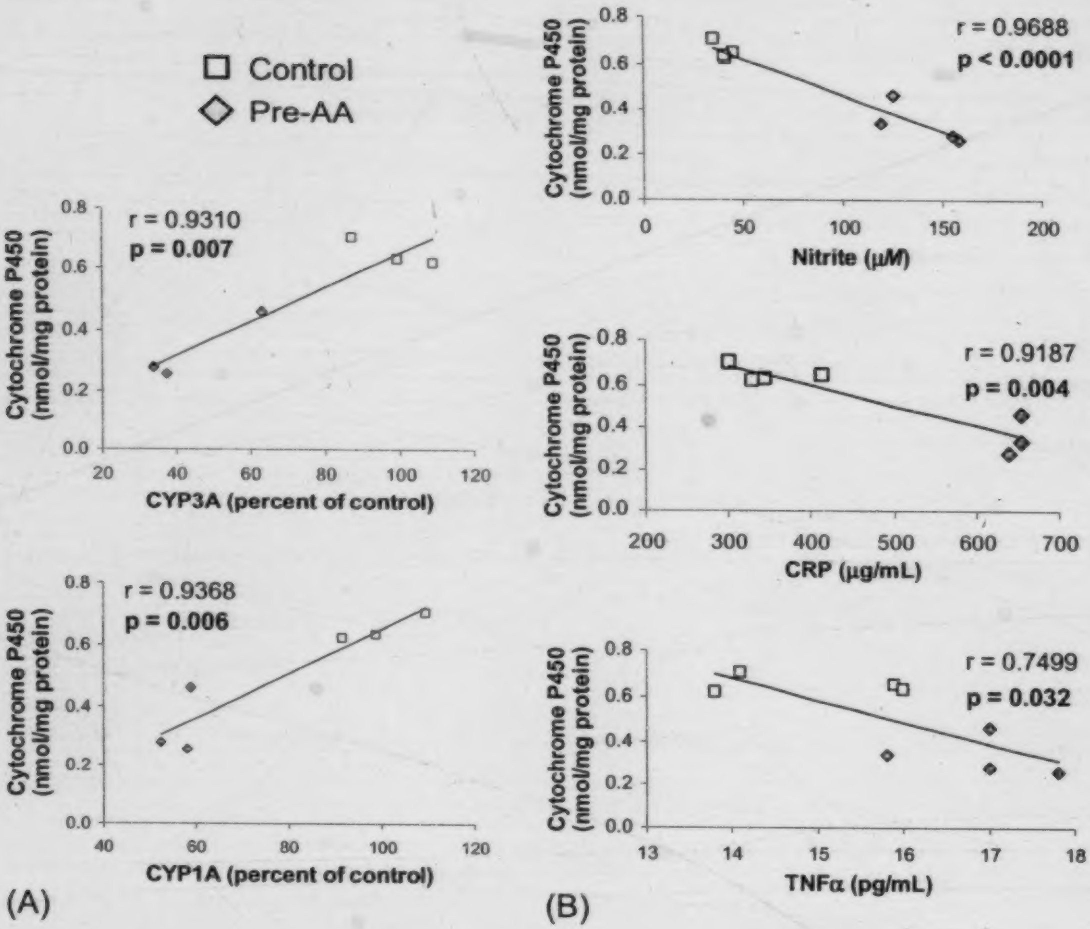


FIGURE 3-5. The correlation between total cytochrome P450 content and: (A) CYP3A and CYP1A expression; and (B) serum nitrite, C-reactive protein, and plasma TNFα concentrations. Regression lines are depicted.

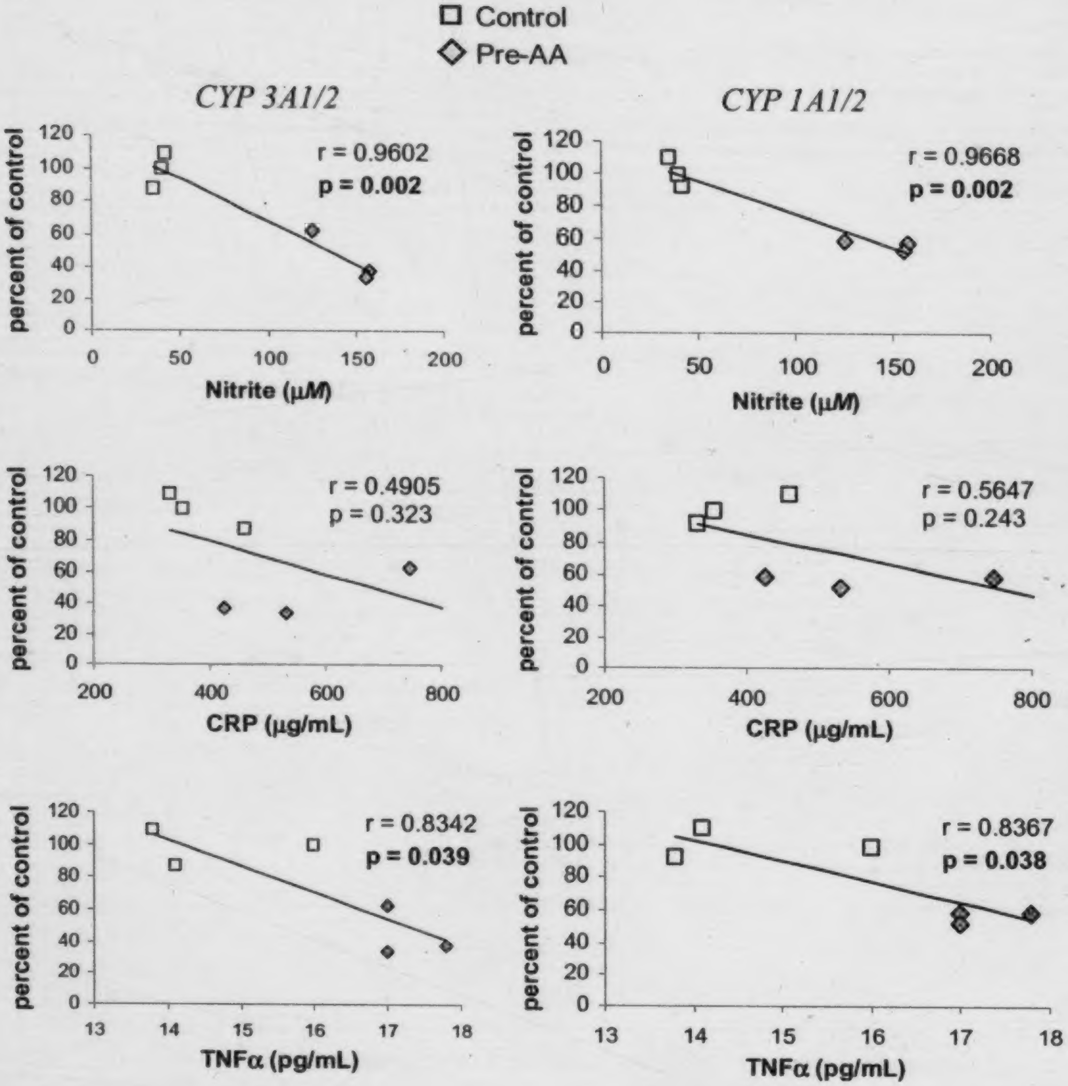


FIGURE 3-6. The correlation between CYP3A1/2 and CYP1A1/2 protein expression and serum nitrite, C-reactive protein and plasma TNFα concentrations. Regression lines are depicted.

Discussion

The adjuvant arthritis (AA) model of chronic inflammation is characterized by elevated pro-inflammatory mediators (Philippe, et al., 1997; Szekanecz, et al., 2000) and development of swelling, pain and deformity of joints 12 to 14 days post-adjuvant (Philippe, et al., 1997; Whitehouse, 1988). AA-induced inflammation also results in increased levels of acute-phase proteins, impaired drug metabolizing enzymes, and thereby reduced clearance of a variety of drugs (Belpaire, et al., 1989; Emami, et al., 1998; Piquette-Miller and Jamali, 1993; Pollock, et al., 1989; Walker, et al., 1986). Although a widely used model, AA is associated with excessive pain and discomfort (Nagakura, et al., 2003). Pre-AA can be an alternative approach to AA. However, for the model to be useful for pharmacokinetic studies, at least, two characteristics are essential: First, the concentration of pro-inflammatory mediators should increase during the early phase of AA development and before full development of the experimental disease. Second, the time lapse between the rise in the mediators concentration and their expected effect on pharmacokinetic indices cannot be greater than the time between the adjuvant injection and the full development of the disease. The pre-AA model appears to meet both these criteria: It causes a substantial rise in CRP and nitrite in a few days (Figure 3-1) and results in reduction in total cytochrome P450, CYP3A and CYP1A content 6 days after the adjuvant injection (Figures 3-4 and 3-6). Consequently, a significant decrease in clearance of verapamil is observed on day 6 (Figures 3-2 and 3-3). Our observation confirms earlier reports that indicate an acute inflammatory response and rises in pro-inflammatory mediators within the first few days of the adjuvant injection (Hirschelmann, et al., 1990; Philippe, et al., 1997; Szekanecz, et al., 2000). In addition, we have shown that this early rise in pro-inflammatory mediators coincides with an increased extent of protein binding for verapamil (Table 3-1) and correlates with a reduction in the total cytochrome P450, CYP3A and CYP1A protein content (Figures 3-4 and 3-6) in as early as 6 days.

Verapamil is a basic drug that is highly bound to the acute-phase protein AAG and is efficiently cleared in the liver. In inflammatory conditions, AAG is increased (Belpaire, et al., 1982) resulting in a greater extent of binding hence reduced free fractions of the drug (Laethem, et al., 1994). For highly protein bound drugs, even small changes in protein binding can result in large relative increases in unbound fraction. In our study, we observed significantly decreased unbound fractions for both enantiomers of verapamil in pre-AA rats as compared to control rats. Other models of inflammation have shown varying effect on protein binding of verapamil. Endotoxin induced inflammation reduces unbound fraction of verapamil (Laethem, et al., 1994) whereas IFNα-2a induced inflammation does not significantly affect protein binding (Sattari, et al., 2003). This suggests that altered protein levels may be dependent on the type of inflammation.

Following iv administration, clearance of highly extracted drugs such as verapamil is dependent primarily on hepatic blood flow (Q) (Gibaldi and Perrier, 1982). Since Q is unaffected by inflammation (Walker, et al., 1986), the significant reduction in clearance of verapamil observed in our study indicates that pre-AA causes substantial reductions in intrinsic metabolic activity consistent with the observed reduction in CYP protein content. Suppression of metabolic activity hence intrinsic clearance may reduce drug clearance to such an extent that it becomes dependent on drug free fraction and intrinsic metabolic activity in addition to Q as with intermediately extracted drugs (Gibaldi and Perrier, 1982). Pre-AA decreased intrinsic clearance of S-verapamil by 70% and R-verapamil by 62%. The apparent extraction ratio (E) determined by the relationship, $E = CL/Q$, demonstrates that E for R-verapamil decreased from 0.96 to 0.76, and for S-verapamil from 0.96 to 0.49. E of S-verapamil therefore, is consistent with values associated with intermediately cleared drugs.

Interestingly, the clearance of propranolol, another highly cleared drug with high affinity for AAG, although reduced by adjuvant arthritis after oral doses, it is not affected after iv dosing (Piquette-Miller and Jamali, 1993). Metabolism of propranolol, which occurs through various enzyme pathways including glucuronidation, may be affected less in inflammation than the metabolism of verapamil which is metabolized primarily by the CYP 3A and 1A isoenzymes (Kroemer, et al., 1993), and therefore remained highly cleared even in the presence of inflammation.

Verapamil is subject to extensive first pass metabolism after oral administration and pharmacokinetic indices are thus influenced by extent of protein binding and alterations in hepatic metabolism. Pre-AA significantly elevated Cmax and AUC_{0-4h} for both enantiomers of verapamil (Table 3-1). Similar effects have been observed in acute inflammation (Laethem, et al., 1994;

Sattari, et al., 2003) and confirm pre-AA is a suitable alternative model of inflammation for pharmacokinetic studies. It is of value to mention that following oral administration to inflamed rats, a greater extent of variability was observed in the plasma verapamil concentrations (Figure 3-3). This is consistent with data reported for verapamil in humans (Mayo, et al., 2000) and propranolol in the rat (Piquette-Miller and Jamali, 1995). The inflammation induced-reduced clearance of these highly cleared drugs depends on the severity of the disease. Hence, the variability in concentrations observed in our inflamed rats is likely a reflection of the inherent inter-subject variability of the severity of the inflammation.

Reduced cytochrome P450 has been reported in various models of inflammation. There are few direct or indirect data on liver metabolic activity in the early phase of AA. During the acute phase of AA-induced inflammation, prolonged sleep time has been reported for pentobarbital (Dipasquale, et al., 1974) and hexobarbital (Baumgartner, et al., 1974) possibly due to decreased drug metabolism. On the other hand cytochrome P450 content appears to be unaltered at day 5 post-adjuvant injection (Morton and Chatfield, 1970) and disposition of cyclosporine after 5 days of AA remains unaffected (Pollock, et al., 1989). Our study showed a 48% reduction in total cytochrome P450 content on day 6 and corresponding decreases in verapamil intrinsic clearance. Importantly, the expression of the specific isoenzymes involved in verapamil metabolism, CYP3A1/2 and CYP1A1/2, were also down-regulated, by 55% and 44%, respectively in by pre-AA. Furthermore, suppression of total cytochrome P450 was significantly correlated to decreases in both CYP3A and CYP1A isoenzymes.

The early rise in pro-inflammatory mediators in pre-AA may have been responsible for the observed reductions in CYP content and verapamil clearance. Serum CRP concentrations were significantly correlated with the decrease in total P450 content, but not with either of the CYP isoforms, suggesting that this acute phase protein is reflective of systemic inflammatory processes but is not directly involved in CYP down-regulation. TNFα levels were only slightly elevated on day 6 but were positively correlated with the down-regulation of both CYP isoforms as well as total P450 content. This suggests that the pro-inflammatory cytokine TNFα has a role in the suppression of CYP enzymes in pre-AA, either directly or indirectly, although the mechanism cannot be inferred by this study. Of the pro-inflammatory mediators measured in this study, serum nitrite levels had the strongest correlation with the down-regulation of CYP3A and CYP1A isoforms, as well as total cytochrome P450 content. Pro-inflammatory cytokines have been shown to suppress cytochrome P450 enzyme activity and content (Morgan, 1997) perhaps through cytokine mediated increase in production of NO, known to inactivate CYP450 enzymes (Khatsenko, et al., 1993). Serum nitrite, a surrogate marker of NO has been positively correlated with arthritic disease severity and inflammation-induced reductions in drug clearance (Mayo, et al., 2000).

In summary, the early phase of AA is marked by increased pro-inflammatory mediators and reduced verapamil clearance as well as decreased hepatic CYP protein content. Hence, pre-AA is a suitable model of inflammation for pharmacokinetic studies that avoids unnecessary exposure of animals to the pain and distress of fully developed adjuvant arthritis.

CHAPTER 4

Exploratory dose-effect study of the effect of infliximab on cytochrome P450 and oral pharmacokinetics of verapamil in pre-AA rats.

Introduction

Clearance of verapamil is inhibited within days of adjuvant inoculation in pre-AA coinciding with increases in pro-inflammatory mediator levels, increased protein binding and reductions in hepatic CYP enzyme expression (Ling and Jamali, 2005). Increased serum levels of pro-inflammatory mediators and acute phase proteins are associated with disease severity (Mayo, et al., 2000; Nakamura, et al., 1993) and are correlated with reduced drug clearance (Piquette-Miller and Jamali, 1995; Sattari, et al., 2003) and decreased hepatic CYP enzyme levels (Ling and Jamali, 2005). Infliximab is a chimeric anti-TNFα antibody that binds and inactivates TNFα, causing a reduction in pro-inflammatory mediators (Charles, et al., 1999; Schuerwegh, et al., 2003). In the acutely inflamed rat, infliximab has also been shown to reverse the down-regulated response to sotalol as well as reduce serum concentrations of nitrite and TNFα (Kulmatycki, et al., 2001). Since inflammation may be responsible for reduced drug clearance (Kulmatycki and Jamali, 2001), we hypothesized that infliximab treatment would reverse the effects of inflammation on drug metabolism and clearance.

It is known that the onset of therapeutic action of infliximab in humans is rapid and its duration of effect long, however, the dose that can effectively influence hepatic enzymes is unknown. The half-life of CYP1A2 proteins is 35 h and for CYP3A4 is 37 h and enzyme induction studies indicate that maximum induction may not be reached for at least 48 h (Muntane-Relat, et al., 1995; Shiraki and Guengerich, 1984). In order to study the effect of infliximab on cytochrome P450 and thereby pharmacokinetics of verapamil, therefore, we conducted a preliminary investigation in which we measured cytochrome P450 content in hepatic microsomes of pre-AA rats 4 days after treatment with 6 mg/kg infliximab and 4, 6, and 8 days after treatment with 10 mg/kg infliximab. We also compare verapamil pharmacokinetics following oral single doses before and after infliximab administration. This was a three-arm parallel study comparing indices in inflamed rats treated with infliximab with healthy control rats.

Methods and Materials

Chemicals

Infliximab (Remicade, Centocor Inc., Malvern, Pennsylvania) was purchased from Schering Canada (Pointe-Claire, Quebec). Verapamil hydrochloride, (+) glaucine, heptafluorobutanol, a*spergillus* nitrate reductase (10 U mL^{-1}), HEPES, FAD, NADPH, lactic dehydrogenase (1500 U mL^{-1}), pyruvic acid, sulfanilamide, naphthylethylenediamine dihydrochloride, sucrose, potassium chloride, sodium chloride, bovine serum albumin, and Folin-Phenol reagent were purchased from Sigma-Aldrich (St. Louis, MO). Sodium dithionite and calcium chloride dihydrate were purchased from BDH (Toronto, ON, Canada). Copper sulfate was purchased from MP Biomedicals (Irvine, CA). Sodium potassium tartarate and sodium bicarbonate were purchased from Fisher Scientific Co. (Pittsburgh, PA). High-performance liquid chromatography (HPLC) grade hexane, HPLC grade isopropanol, triethylamine (TEA), and 98% ethanol were purchased from Caledon Laboratories (Georgetown, ON, Canada). Heat-killed, dried *Mycobacterium butyricum* was purchased from Difco (Detroit, MI). Rat C-Reactive Protein ELISA kit was purchased from Helica Biosystems Inc. (Fullerton, CA). Rat TNFα Ultrasensitive ELISA kit was purchased from BioSource International (Camarillo, CA).

Animals

Experiments were performed on male, Sprague-Dawley rats (250-300 g), and were approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Animals had free access to water but food was withheld for 12 h prior to pharmacokinetic experiments. They were housed under a 12 h light/dark cycle.

All invasive experimental procedures were performed while rats were under halothane/O_2 (5/95%) anesthesia administered via vaporizer and circuit mask.

The right jugular vein was cannulated under anesthesia as follows. Briefly, a polyethylene (PE-50; Clay Adams, Parsippany, NY) cannula tipped with 2 cm of silastic (Dow Corning Corp, Midland, MI) tubing was inserted into the right jugular vein and exteriorized by subcutaneous tunneling to an incision made in the interscapular area.

Protocol

The time course of experimental treatments is depicted in Figure 4-1. The day of adjuvant injection (0.2 mL of 50 mg/mL *Mycobacterium butyricum* suspended in squalene into the tail base) was marked as day 0. Control animals received 0.2 mL saline into the tail base. On day 5, the right jugular vein was cannulated for serial blood collection. On day 6, pre-AA/infliximab rats were administered infliximab at doses of 3 mg/kg (n=4), 6 mg/kg (n=4-6), or 10 mg/kg (n=9) by intravenous injection. Healthy control (n=3-6) rats received an equal volume of sterile normal saline.

Effect of 3 mg/kg (Study A) and 6 mg/kg (Study B) infliximab on oral verapamil pharmacokinetics

Rats receiving 3 mg/kg infliximab (n=4) and healthy control rats (n=6) were administered single doses of racemic verapamil orally via gastric gavage (20 mg/kg suspended in polyethylene glycol 400) 2 days after infliximab treatment. Blood samples (~0.2 mL) were collected at 0, 0.25, 0.5, 0.75, 1, 2 and 4 h after oral doses. Plasma was separated and kept at -80° C for stereospecific verapamil assay.

Rats receiving 6 mg/kg infliximab (n=4) and healthy control rats (n=3) were administered single doses of racemic verapamil orally via gastric gavage (20 mg/kg suspended in polyethylene glycol 400) 4 days after infliximab treatment. Blood samples (~0.2 mL) were collected at 0, 0.25, 0.5, 0.75, 1, 2, 4, 6 and 8 h after oral doses. Plasma was separated and kept at -80° C for stereospecific verapamil assay.

For rats receiving 6 mg/kg infliximab, blood samples were also taken on days 6 and 10 for the determination of serum nitrite, C-reactive protein and plasma TNFα concentrations.

Effect of 6 mg/kg (Study B) and 10 mg/kg (Study C) infliximab on cytochrome P450 content

Rats receiving 6 mg/kg infliximab were sacrificed following pharmacokinetic experiments on day 10, and their livers removed for microsomal preparation. Rats receiving 10 mg/kg infliximab were not cannulated and did not receive verapamil. These animals received infliximab on day 6 and were sacrificed on days 10, 12, or 14 (n=3/group), and their livers removed for microsomal preparation.

Microsomal Preparation

Immediately after sacrifice, rat livers were removed and hepatic microsomes prepared (Barakat, et al., 2001). Briefly, livers were rinsed in 0.15 *M* KCl solution and homogenized in 0.25 *M* sucrose solution. The homogenates were centrifuged at 12000 *g* at 4° C for 10 min. The S9 fraction was collected and microsomes precipitated by addition of 1 *M* $CaCl_2$. The suspension was centrifuged at 27000 *g* at 4° C for 15 min, then the pellet was re-suspended in

0.15 *M* KCl solution and centrifuged at 27000 *g* at 4° C for 15 min. The pellet was again re-suspended in 0.25 *M* sucrose solution and stored at -80° C.

The microsomal protein concentration was determined by the Lowry method (Lowry, et al., 1951). Briefly, microsomes was incubated with 1% $CuSO_4$:2% Na.K.Tartarate:10% Na_2CO_3 anhydrous in 0.5 *M* NaOH (1:1:20 v/v/v) at room temperature for 10 min, then with 10% Folin-Phenol reagent at 50° C for 10 min. The sample was analyzed by spectrophotometry at 570 nm.

Total cytochrome P450 content was determined by the method of Omura and Sato (Omura and Sato, 1964). Briefly, microsomes were suspended in 0.05 M phosphate buffer, pH 7.4, at a protein concentration of 1 mg/mL. Ten milligrams of solid sodium dithionite were added to the 1 mL of suspension and a baseline determined using the recording spectrophotometer by scanning from 500 to 400 nm. Carbon monoxide was bubbled gently into the sample cuvette for 20 sec. The spectrum was again recorded from 500 to 400 nm. The quantity of cytochrome P450 was calculated from the optical density difference (450-480 nm) and the molar extinction coefficient of 91 $mM^{-1}cm^{-1}$.

Stereospecific Verapamil assay

A stereospecific high performance liquid chromatography (HPLC) method (Shibukawa and Wainer, 1992) was used to determine plasma concentrations of R and S verapamil. Briefly, 75 µl of (+)-glaucine (400 ng/mL) as internal standard was added to 100 µl plasma, followed by 100 µl of 2 M NaOH and 0.4 mL sodium phosphate buffer (pH 7.0, ionic strength 0.1). Verapamil was extracted with 6 mL heptane:heptafluorobutanol (99:1) and vortex mixed, followed by centrifugation. The organic layer was evaporated to dryness, the residue reconstituted in mobile phase (hexane-isopropanol-ethanol-TEA, 92:4:4:0.1v/v/v/v) and injected into an isocratic HPLC system at a flow rate of 0.7 mL/min. The HPLC apparatus consisted of a Waters WISP 712 autoinjector (Milipore-Waters, Mississauga, Canada), an achiral column (5 cm x 4.6 mm I.D. Supelcosil LCSi, Supelco Inc., Bellefonte, PA) and chiral column (250 mm x 4.6 mm I.D., 5 µm Chiralpak AD-H column, Daicel Chemical Inc., Tokyo, Japan) maintained at 31°C, a 474 fluorescence detector (Waters, Mississauga, Canada) set at excitation of 272 nm and emission at 317 nm with a bandwidth at 18 nm, and a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA). Standard curves were linear over the concentration range of 10 – 1000 ng/mL ($r^2 \geq 0.99$, cv $\leq$10%). The minimum quantifiable concentration was 5 ng/mL for S- and R-verapamil.

Serum Nitrite Analysis

Serum nitrite (NO_2^-), a stable breakdown product of nitric oxide (NO), was measured using a previously described method (Grisham, et al., 1996). Briefly, nitrate (NO_3^-) was reduced to nitrite (NO_2^-) by incubating 100 µl of serum with 10 µl of *Aspergillus* nitrate reductase (10 U/mL) in the presence of 25 µl 1 *M* HEPES (pH 7.4), 25 µl 0.1 m*M* FAD and 50 µl 1 m*M* NADPH for 30 min at 37° C. Then, 5 µl of lactate dehydrogenase (1500 U/mL) and 50 µl of 100 m*M* pyruvic acid were added and incubated for an additional 10 min at 37° C. NO_2^- was determined by addition of 1.0 mL Griess reagent and absorbance measured at 543 nm. Standard curves were linear over the concentration range of 3 – 200 μ*M* ($r^2 \geq 0.99$, cv ≤ 10%). The minimum quantifiable concentration was 3 μ*M*.

Serum C-reactive Protein Analysis

A commercially available rat-CRP ELISA kit (Helica Biosystems, Inc., Fullerton, CA) was used. This assay required 100 μl of serum (1:10 000 dilution) to be added to a 96 well plate coated with antibodies to rat-CRP. After incubation for 30 min, the plate was washed and 100 μl of Conjugate (horseradish peroxidase (HRP)-labeled rabbit anti-rat CRP-IgG) was added and incubated for 30 min. The plate was again washed and 100 μl of TMB substrate solution was added and incubated for 10 min. Stop Solution (100 μl) was added to stop the reaction and absorbance was read at 450 nm. Standard curves were linear over the concentration range of 17.5 – 133 μg/mL ($r^2 \geq 0.99$, cv ≤ 10%). The limit of detection of the assay was 2.5 μg/mL.

Plasma TNFα Analysis

Plasma TNFα concentrations were measured using a commercially available rat-TNFα ultrasensitive ELISA kit (Biosource International, Camarillo, CA). Briefly, 100 μl of plasma was added to a 96 well plate coated with anti-rat-TNFα capture antibody. After incubation for 3 h, the plate was washed and 100 μl of Biotin Conjugate added and incubated for 45 minutes. The plate was again washed and 100 μl of Streptavidin-HRP added and incubated for 45 minutes.

After washing, 100 μl of Chromogen was added and incubated for 20 minutes followed by addition of 100 μl of Stop Solution and the absorbance read at 450 nm. Standard curves were linear over the concentration range of 2.3 - 150 pg/mL ($r^2 \geq 0.99$, cv $\leq 10\%$). The limit of detection of the assay was 1.9 pg/mL.

Data Analysis and Statistics

Pharmacokinetic indices for S and R verapamil after oral administration were determined by noncompartmental analysis. Elimination rate constants (β) were calculated using log-linear regression of at least three points in the log-linear terminal phase of the plasma concentration-time curve. The area under the plasma concentration-time curve (AUC) was calculated using the log-linear trapezoidal rule from 0 h to the time of the last measured plasma concentration (C_{last}). Extrapolation to infinity ($AUC_{t-\infty}$) was determined by C_{last}/β, and $AUC_{0-\infty}$ was determined as the sum of AUC_{0-t} and $AUC_{t-\infty}$. Oral clearance (CL/F) was calculated as dose/ $AUC_{0-\infty}$ and apparent volume of distribution was calculated as CL/β.

Data are presented as mean ± standard deviation. Statistical significance between pre-AA/infliximab and control groups were determined by the two-tailed Student's t-test at α=0.05. Comparison of total P450 content in pre-AA rats 4, 6 and 8 days following injection of 10 mg/kg infliximab was done by analysis of variance with Tukey's adjustment for multiple comparisons.

Results

As expected pre-AA significantly elevated serum nitrite, CRP and TNFα concentrations. This effect was evident on day 6 following injection of the adjuvant (Figure 4-2). On day 10, 4 days following single 6 mg/kg doses of infliximab, however, CRP and TNFα concentrations were not significantly different from healthy controls. Serum nitrite levels, on the other hand, remained significantly higher than controls in inflamed rats that received infliximab (Figure 4-2).

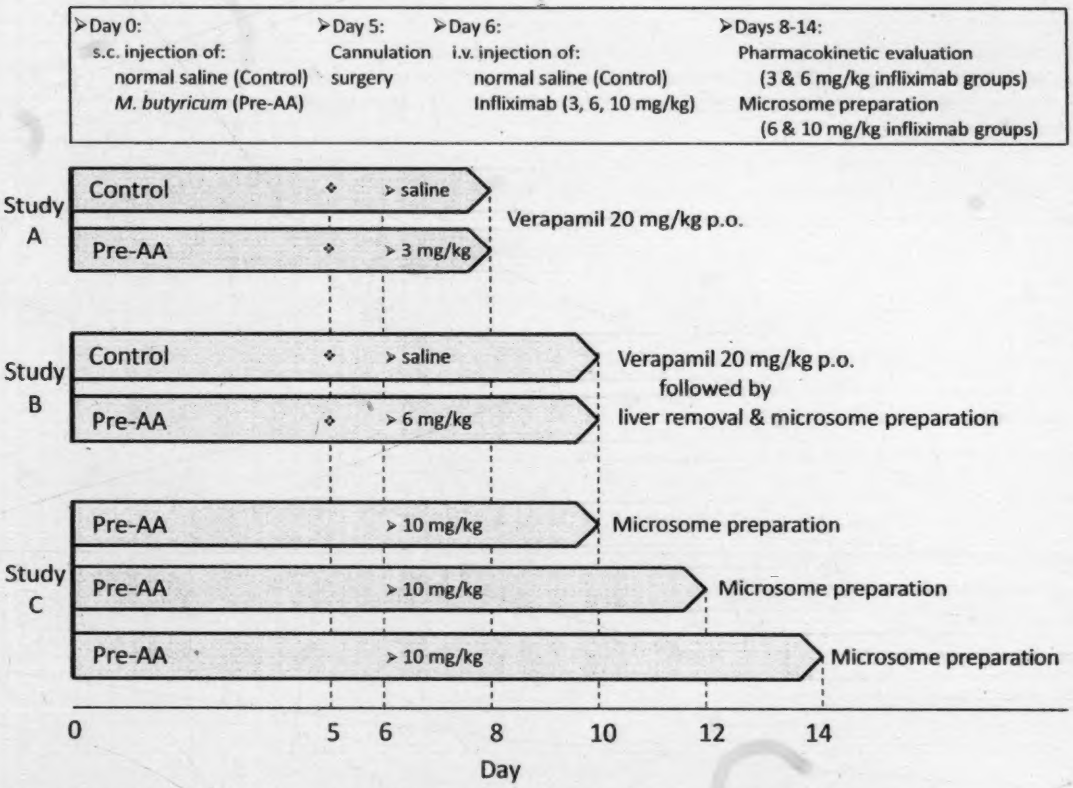
Verapamil enantiomers concentrations were significantly increased by inflammation and did not decline in response to single infliximab doses of 3 mg/kg (Figure 4-3a, Table 4-1). Elevated S- and R-verapamil plasma concentrations were also observed in inflamed rats treated with 6 mg/kg infliximab (Figure 4-3b, Table 4-1) although the increase was not statistically significant. This was due to the small sample size of healthy control rats; nevertheless, the observed verapamil concentrations were consistent with those previously noticed and indicative of reduced verapamil clearance. Blood was collected for only 4 h following verapamil administration in rats treated with 3 mg/kg infliximab. As a result we did not measure enough plasma concentrations in the elimination phase of the concentration time profile to estimate β, CL/F and Vd/F. For rats treated with 6 mg/kg infliximab, verapamil plasma concentrations were determined for up to 8 h and the calculated pharmacokinetic indices listed in Table 4-1. Oral clearance and apparent volume of distribution were significantly reduced in inflamed rats and did not decline in response to infliximab.

Total hepatic microsomal cytochrome P450 content in pre-AA/infliximab rats 4 days after treatment with single 6 mg/kg doses of infliximab (day 10) was lower than in control rats, although this difference did not reach statistical significance (p=0.06) (Figure 4-4). Eight days following injection of single 10 mg/kg doses of infliximab, the cytochrome P450 contents of pre-AA/infliximab rats was significantly elevated as compared with the levels measured 4 and 6 days after treatment (Figure 4-5). The day 4 and 6 values were consistent with those we noticed previously and were indicative of enzymatic suppression (Ling and Jamali, 2005).

TABLE 4-1. Pharmacokinetic indices of verapamil enantiomers following single oral 20 mg/kg doses.

	Study A: effect of infliximab 3 mg/kg after 2 days			*Study B: effect of infliximab 6mg/kg after 4 days*		
	Control (n=4)	Pre-AA /Infliximab (3 mg/kg) (n=6)	Student's t-test p value	Control (n=3)	Pre-AA /Infliximab (6 mg/kg) (n=4)	Student's t-test p value
C_{max}(ng/mL)						
S	356 ± 324	1550 ± 894	0.01	227 ± 144	2285 ± 1683	0.09
R	76 ± 43	286 ± 200	0.02	91 ± 11	475 ± 344	0.12
AUC_{0-t} (μg.min/mL)						
S	54 ± 59	311 ± 208[1]	0.01	41 ± 26	790 ± 631[2]	0.10
R	8 ± 5	46 ± 30	0.01	20 ± 6	150 ± 129	0.15
CL/F (L/min/kg)						
S	-	-	-	0.509 ± 0.350	0.036 ± 0.034	0.04
R	-	-	-	0.650 ± 0.216	0.210 ± 0.176	0.03
Vd/F (L/kg)						
S	-	-	-	168 ± 137	9 ± 6	0.06
R	-	-	-	314 ± 106	71 ± 67	0.01
$t_{1/2}$ (h)						
S	-	-	-	3.6 ± 0.5	3.7 ± 1.2	0.89
R	-	-	-	5.6 ± 0.6	3.5 ± 0.8	0.01

[1] AUC_{0-4h}
[2] AUC_{0-8h}



➢Day 0:
s.c. injection of:
normal saline (Control)
M. butyricum (Pre-AA)
➢Day 5:
Cannulation surgery
➢Day 6:
i.v. injection of:
normal saline (Control)
Infliximab (3, 6, 10 mg/kg)
➢Days 8-14:
Pharmacokinetic evaluation
(3 & 6 mg/kg infliximab groups)
Microsome preparation
(6 & 10 mg/kg infliximab groups)
Study A
Control
➢ saline
Pre-AA
➢ 3 mg/kg
Verapamil 20 mg/kg p.o.
Study B
Control
➢ saline
Pre-AA
➢ 6 mg/kg
Verapamil 20 mg/kg p.o. followed by liver removal & microsome preparation
Study C
Pre-AA
➢ 10 mg/kg
Microsome preparation
Pre-AA
➢ 10 mg/kg
Microsome preparation
Pre-AA
➢ 10 mg/kg
Microsome preparation
0
5
6
8
10
12
14
Day

FIGURE 4-1. Time course of experimental protocol. All rats received adjuvant (*M. butyricum*) or normal saline on day 0. On day 5, rats used for pharmacokinetic experiments were cannulated in the right jugular vein. On day 6, pre-AA rats received infliximab at doses of 3, 6 or 10 mg/kg by iv infusion. Control rats received normal saline. Study A) Rats receiving 3 mg/kg infliximab received verapamil on day 8 (2 days post-infliximab) followed by serial blood collection for 4 h. Study B) Rats treated with 6 mg/kg infliximab received verapamil on day 10 (4 days post-infliximab) followed by serial blood collection for 8 h and then sacrificed and livers removed for microsome preparation. Study C) Rats receiving 10 mg/kg infliximab did not receive verapamil; these rats were sacrificed on day 10, 12, or 14 for liver removal and microsome preparation.

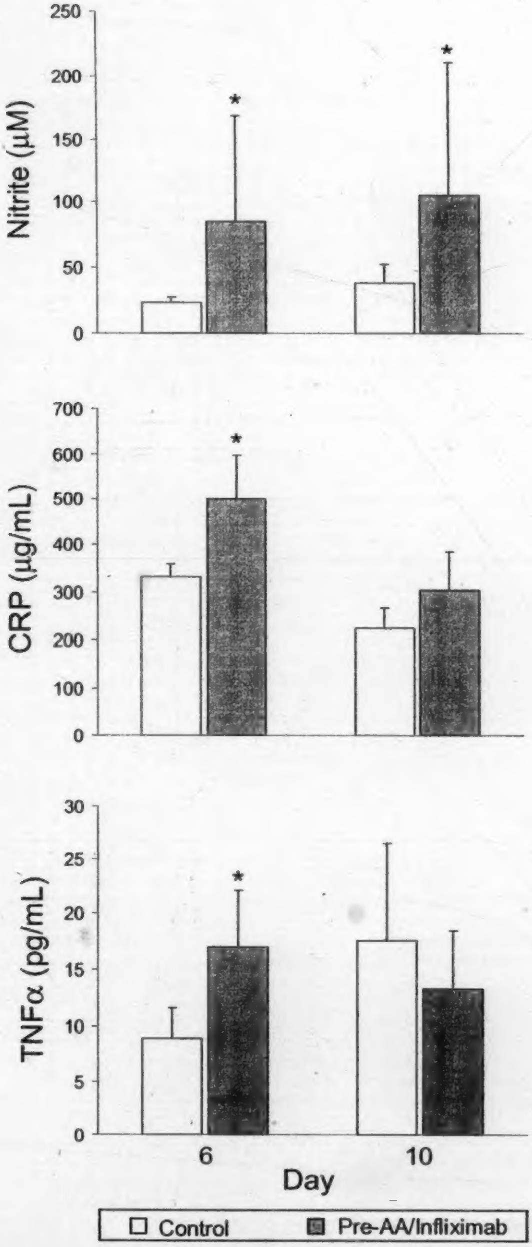
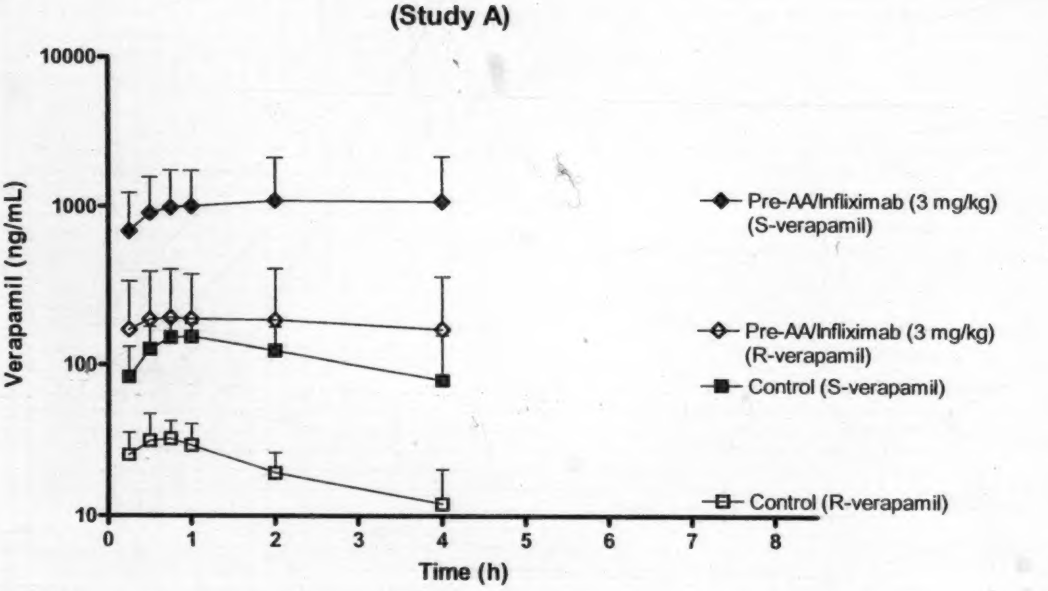


FIGURE 4-2. (Study B) Mean pro-inflammatory mediators concentrations in healthy control and infliximab-treated pre-AA rats. The latter group received single 6 mg/kg doses of infliximab for 4 days (i.e. day 10 post-adjuvant) (n=3-4/group). *$p<0.05$ vs control.



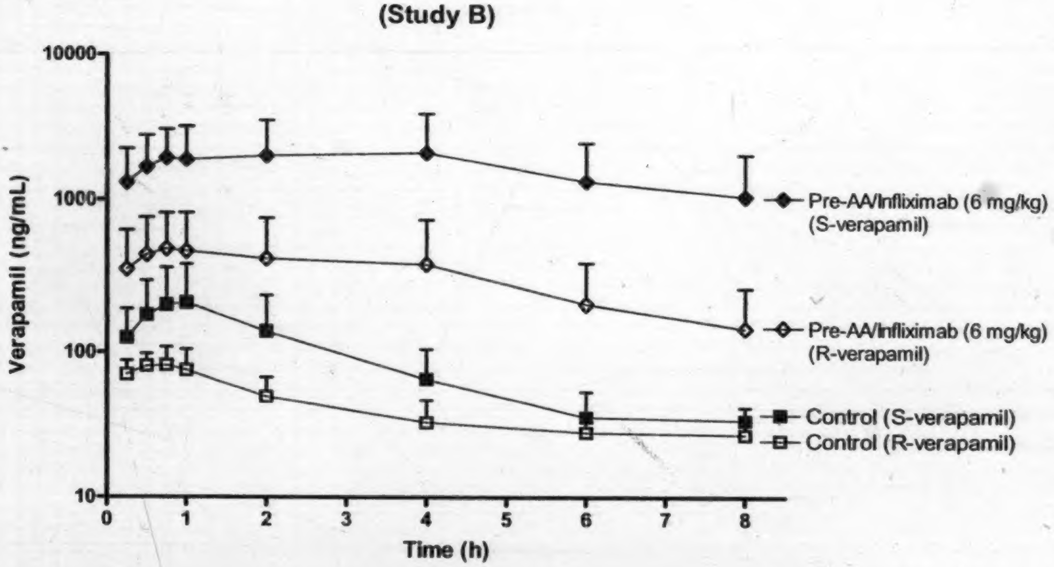


FIGURE 4-3. (Study A) Mean plasma verapamil concentrations following administration of single oral 20 mg/kg racemic verapamil doses to pre-AA/infliximab (3 mg/kg; n=4) and healthy control (n=6) rats 2 days after infliximab dose. Study B) Mean plasma verapamil concentrations following administration of single oral 20 mg/kg racemic verapamil doses to pre-AA/infliximab (6 mg/kg; n=4) and control (n=3) rats 4 days after infliximab dose.

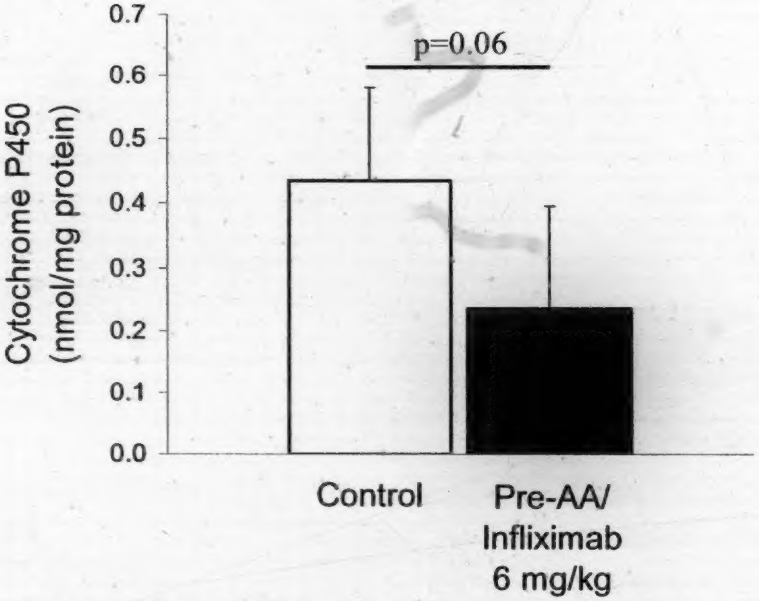


FIGURE 4-4. (Study B) Mean cytochrome P450 content in healthy control (n=5) and pre-AA/infliximab (n=6) rats 4 days following treatment with single 6 mg/kg doses of infliximab.

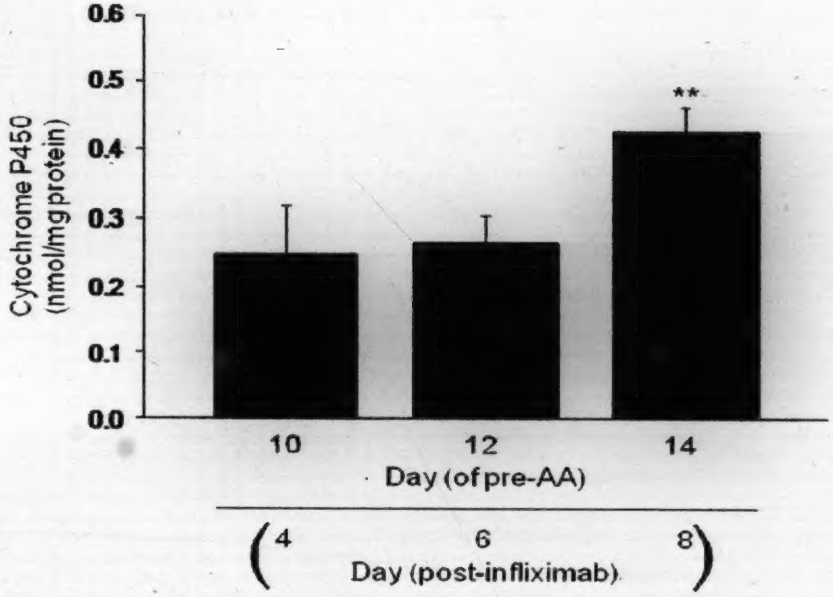


FIGURE 4-5. (Study C) Mean cytochrome P450 content 4, 6 and 8 days following single 10 mg/kg doses of infliximab in pre-AA rats (n=3/group). Error bars represent standard deviation of the means. **$p \leq 0.01$ (ANOVA).

Discussion

We have previously shown that verapamil enantiomers concentrations are elevated in pre-AA (Ling and Jamali, 2005). Interestingly, infliximab at 3 and 6 mg/kg did not appear to result in a decline of verapamil concentrations toward those of healthy control rats as the values were still substantially and significantly higher in pre-AA rats that received infliximab than in healthy controls (Figure 4-3). Due, perhaps, to the small sample size, increases in verapamil concentration among inflamed rats treated with 6 mg/kg infliximab were not statistically significant; however, the plasma concentrations were consistent with those observed in pre-AA. Cytochrome P450 content was decreased in pre-AA rats 4 days after treatment with 6 mg/kg infliximab, although the decrease did not reach significance (p=0.06) (Figure 4-4). Closer inspection of the data revealed the P450 content of one animal to be significantly higher than others in the pre-AA/infliximab group. The P450 level for this animal was a statistical outlier (Grubb's test, p=0.006), perhaps due to lack of response or slow response of the animal to adjuvant injection. Weight gain for this rat was consistent with that of the control animals; however, pro-inflammatory mediators were within the range of other pre-AA/infliximab animals. We therefore did not exclude this animal from the pre-AA/infliximab group for data analysis.

The effect of the higher dose of infliximab, i.e., 10 mg/kg eight days following its injection was associated with a significant rise in the cytochrome P450 contents of the liver microsomes compared with day 4 and day 6 levels (Figure 4-5).

In pre-AA, decreased clearance of verapamil coincides with elevated concentrations of pro-inflammatory mediators nitrite, CRP and TNFα. Furthermore, increased expression of nitrite and TNFα is significantly correlated with suppression of total cytochrome P450 and CYP1A and CYP3A enzyme content (Ling and Jamali, 2005). In this preliminary study, the rise in pro-inflammatory mediators 6 days after injection of adjuvant was expected (Figure 4-2). Four days after single doses of 6 mg/kg infliximab, serum CRP and TNFα concentrations appeared reduced to healthy control levels (Figure 4-2). We suspect this to be due to the effects of infliximab, which has been shown to reduce CRP and TNFα in RA patients (Charles, et al., 1999; Schuerwegh, et al., 2003). We did not include an untreated inflamed group; therefore, we cannot conclude whether the apparent decrease in CRP and TNFα levels was due to the effects of infliximab or due to natural progression of pre-AA. However, since pre-AA is expected to progress to a more chronic and severe inflammatory disease, as symptomatic AA develops, continued elevations in serum CRP and TNF would be anticipated in the absence of infliximab treatment.

Surprisingly, serum nitrite levels remained significantly elevated in inflamed rats 4 days after treatment with single doses of 6 mg/kg infliximab (Figure 4-2). Although rat specific anti-TNFα antibodies have been shown to suppress the development of experimental arthritis in animals (Bush, et al., 2002; Williams, et al., 2000), the effect of infliximab, a 75% human and 25% murine chimeric monoclonal antibody, on experimental arthritis in the rat has not been established. We studied the effect of infliximab on hepatic microsomal CYP content and verapamil pharmacokinetics after the documented rise in pro-inflammatory mediators but before the development of arthritic symptoms. Treatment with anti-TNFα after the development of inflammation, rather than at the time of adjuvant induction, is more relevant to human disease and it has been shown that anti-TNFα antibody can reduce the clinical severity of established arthritic disease in mice (Williams, et al., 1992; Williams, et al., 2000). In non-arthritic disease models, infliximab has been shown to decrease inflammation and disease activity (Olmarker, et al., 2003; Woodruff, et al., 2003). Most relevant to our investigation was a study by Kulmatycki et al., showing a reduction in serum nitrite and TNFα levels 8 h after a single 3 mg/kg dose of infliximab in the acutely inflamed rat. In addition to reduction in pro-inflammatory mediators, infliximab also reversed the effects of acute inflammation on both PR and QT intervals in the rat (Kulmatycki, et al., 2001). The elevated levels of serum nitrite in our study may have been caused by cytokine-mediated T cell function, which has been shown not to be suppressed by TNFα blockade in early phase of AA (Bush, et al., 2002). Nevertheless, the persistent rise in serum nitrite may explain the continued suppression of hepatic P450 content and altered verapamil pharmacokinetics following single doses of 6 mg/kg infliximab in our study. Nitric oxide has been shown to inactivate cytochrome P450 enzymes (Khatsenko, et al., 1993) and perhaps mediates cytokine-induced suppression of CYP450 activity and content (Morgan, 1997). In pre-AA, down-regulation of hepatic CYP enzymes is strongly correlated with increases in nitrite (Ling and Jamali, 2005).

Besides specificity, infliximab dose and route of administration has not been studied in rats. For RA, recommended infliximab doses are 3 to 10 mg/kg. Since infliximab must be reconstituted at a concentration of 10 mg/mL, administration of 20 to 70 mL are required. Such large volumes are unsuitable for subcutaneous administration, hence infliximab is only given by iv infusions. High concentration preparations of monoclonal antibodies may be an alternative, however, such highly concentrated solutions reportedly result in very high viscosity, protein aggregation and poor overall stability (Yang, et al., 2003). Studies reported in the literature involving the use of infliximab in the rat have been done with iv or ip administration at doses of between 3 to 10 mg/kg. For this study, we compared doses of between 3 and 10 mg/kg which we administered intravenously. In rats, infliximab doses of up to 10 mg/kg require volumes of less than 0.5 mL. Therefore, in future studies, subcutaneous injection may be used, since this route of administration has been shown to have similar bioavailability and pharmacokinetic profile as iv administration in rats (Yang, et al., 2003). The half life of elimination of infliximab is approximately 12 to 16 days in rats following iv or sc administration (Yang, et al., 2003), so infliximab is expected to still be in circulation at the time we performed our experiments. Because infliximab comprises human and murine amino acid sequences, there is the possibility of the development of rat anti-chimeric antibodies. In RA patients, the formation of human anti-chimeric antibodies decreases as infliximab dose is increased (Maini, et al., 1998). Formation of autoantibodies is associated with reduced therapeutic response, due to inactivation of infliximab, and increased clearance of infliximab/antibody complex. Although we cannot rule out this possibility as an explanation for the lack of effect observed in our study, it is unlikely for an autoantibody response to be a significant factor following a single dose of the drug.

Another important consideration is the length of time required for down-regulated hepatic CYP enzymes to recover following infliximab treatment. Although infliximab may reduce inflammation within hours of a dose (Kreutz, et al., 2004; Kulmatycki, et al., 2001; Oruc, et al., 2004), hepatic enzyme recovery may require days, particularly if the down regulation is at the transcription level. Studies on the inducible expression of CYP's 1A1, 1A2 and 3A4 in human hepatocytes indicate that maximum induction is reached at 12 to 30 h for mRNA and at least 48 h for CYP proteins, in addition to protein half-life of 35 to 37 h (Muntane-Relat, et al., 1995; Shiraki and Guengerich, 1984). We therefore examined the effect of time following an infliximab injection on cytochrome P450 content in pre-AA rats (Figure 4-5). Figure 4-5 shows a significant rise in hepatic P450 contents 8 days following single doses of 10 mg/kg infliximab compared with day 4 and day 6 levels. Cytochrome P450 levels 4 to 6 days after infliximab treatment were consistent with suppressed levels observed in pre-AA rats. Our results suggest a time delay of at least 8 days between infliximab treatment and recovery of hepatic enzymes. We do not know if a longer time period would have resulted in even higher P450 enzyme levels, since we limited our study to the pre-arthritic phase of disease. It is also not known whether the recovery of P450 enzymes 8 days after infliximab injection will influence pharmacokinetics of verapamil.

In summary, plasma concentrations of verapamil were elevated in pre-AA rats despite treatment with infliximab at doses of 3 and 6 mg/kg, due perhaps, to suppression of hepatic cytochrome P450. Infliximab appears to reduce plasma concentrations of CRP and TNFα, but not nitrite. Infliximab at a dose of 10 mg/kg significantly increased CYP450 levels 8 days after treatment.

CHAPTER 5

The effect of infliximab on hepatic CYP enzymes and pharmacokinetics of verapamil in pre-adjuvant arthritis rats.

Introduction

Clearance of verapamil is reduced in patients with rheumatoid arthritis and in the pre-adjuvant arthritis model (pre-AA) of inflammation in the rat, resulting in elevated plasma drug concentrations (Ling and Jamali, 2005; Mayo, et al., 2000). Both rheumatoid arthritis and pre-AA causes increased expression of pro-inflammatory mediators which are associated with suppression of cytochrome P450 enzymes (Ling and Jamali, 2005; Mayo, et al., 2000) and hence, reduced clearance of efficiently cleared drugs (Ling and Jamali, 2005; Piquette-Miller and Jamali, 1992; Walker, et al., 1986).

Rheumatoid arthritis is associated with a high prevalence of cardiovascular disease (Maradit-Kremers, et al., 2005a; Maradit-Kremers, et al., 2005b; McEntegart, et al., 2001; Myllykangas-Luosujarvi, et al., 1995; Solomon, et al., 2003; Wolfe, et al., 1994) in association with the presence of elevated pro-inflammatory cytokines that underlie the pathogenesis of both conditions (Pasceri and Yeh, 1999; Sattar, et al., 2003). In the rat, adjuvant arthritis (AA) has also been shown to adversely affect the cardiovascular system. Reduced cardiac response to the drugs propranolol, sotalol, and verapamil has been demonstrated in rats with AA, perhaps as a result of inflammation-induced suppression of β-adrenergic, potassium and calcium channel function (Guirguis and Jamali, 2003;

Kulmatycki, et al., 2001; Sattari, et al., 2003). Interestingly, the down-regulated response to sotalol is reversed in acutely inflamed rats treated with infliximab, an anti-TNFα antibody that binds and inactivates TNFα, causing a reduction in pro-inflammatory mediators (Charles, et al., 1999; Kulmatycki, et al., 2001; Schuerwegh, et al., 2003). This intriguing finding supports the concept of inflammation as mediator of altered drug response. Since inflammation is also implicated in reduced drug clearance (Kulmatycki and Jamali, 2001), we hypothesized that infliximab treatment would also reverse the effects of inflammation on drug metabolism and clearance. In this study, we examined hepatic cytochrome P450 content and pharmacokinetics of verapamil in AA rats treated with infliximab during the pre-arthritic (pre-AA) phase of disease. Pre-AA is the early phase of AA when systemic inflammatory changes have already taken place before the manifestation of disabling physical symptoms of AA such as swelling of the joints (Ling and Jamali, 2005).

Materials and Methods

Chemicals

Infliximab (Remicade®, Centocor Inc., Malvern, Pennsylvania) was purchased from Schering Canada (Pointe-Claire, Quebec). Verapamil hydrochloride, (+) glaucine, heptafluorobutanol, a*spergillus* nitrate reductase (10 U mL^{-1}), HEPES, FAD, NADPH, lactic dehydrogenase (1500 U mL^{-1}), pyruvic acid, sulfanilamide, naphthylethylenediamine dihydrochloride, sucrose, potassium chloride, sodium chloride, bovine serum albumin, and Folin's-Phenol reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dithionite and calcium chloride dihydrate were purchased from BDH Chemicals (Toronto, Canada). Copper sulfate, Tris (0.025 *M*)/Glycine (0.192 *M*) Buffer, and Tris (0.025 *M*)/Glycine (0.192 *M*)/SDS (0.1%) Buffer was purchased from ICN Biomedicals Inc. (Aurora, OH). Sodium potassium tartarate, sodium bicarbonate, and Tween-20 were purchased from Fisher Scientific (Fair Lawn, NJ). Tris was purchased from Invitrogen (Carlsbad, CA). Ammonium persulfate, electrophoresis grade, was purchased from BioShop Canada Inc., (Burlington, Canada). Sodium azide, TEMED, and 2-mercaptoethanol were purchased from EM Science (Gibbstown, NJ). Sodium dodecyl sulfate, Laemmli sample buffer and 40% Acrylamide/Bis solution were purchased from BioRad Laboratories (Hercules, CA). High-performance liquid chromatography (HPLC) grade hexane and HPLC grade isopropanol, triethylamine (TEA), and 98% ethanol were purchased from Caledon Laboratories (Georgetown, Canada). Heat-killed, dried *Mycobacterium butyricum* was purchased from Difco Laboratories (Detroit, MI). Rat C-Reactive Protein ELISA kit was purchased from Helica Biosystems Inc. (Fullerton, CA).

Animals

Experiments were performed on male, Sprague-Dawley rats (250-300 g), and were approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Animals had free access to water but food was withheld for 12 hours prior to pharmacokinetic experiments. They were housed under a 12 hour light/dark cycle.

Rationale for using Pre-AA

The pre-AA model of inflammation (Ling and Jamali, 2005) was used to minimize pain and stress associated with AA. Pre-AA has been shown to increase levels of pro-inflammatory mediators TNFα, CRP and nitrite within days of adjuvant injection, and is associated with decreased oral and systemic clearance of verapamil. In addition, increased pro-inflammatory mediators were significantly correlated with the suppression of hepatic CYP enzymes in pre-AA. Moreover, all of these inflammation-induced effects were observed in the absence of the pain and stress associated with the experimental disease. Our aim was to study the effect of infliximab on hepatic microsomal CYP content and verapamil pharmacokinetics after the documented rise in pro-inflammatory mediators but before the development of arthritic symptoms. Treatment with anti-TNFα after the development of inflammation, rather than at the time of adjuvant induction, is more relevant to human disease and it has been shown that anti-TNFα antibody can reduce the clinical severity of established arthritic disease in mice (Williams, et al., 1992; Williams, et al., 2000).

Inflammation Biomarkers

Serum nitrite is a reliable predictor of the severity of rheumatoid arthritis in humans (Mayo, et al., 2000) and clearance of verapamil in rats with inflammation (Ling and Jamali, 2005; Sattari, et al., 2003). TNFα concentrations, as measured using the commercially available assay kits (Ling and Jamali, 2005; Sattari, et al., 2003), however, exhibit great variability so that their usefulness are limited. In addition, the available TNFα ELISA assays measure monomeric and trimeric TNFα as well as TNFα bound and unbound to either of two TNFα receptors (De Groote, et al., 1993; Engelberts, et al., 1991; MacEwan, 2002). Consequently, serum levels of TNFα measured by immunoassay may not reflect the biologically active cytokine (Corti, et al., 1992). For example, in human serum, infliximab treatment results in significantly higher circulating levels of immunoreactive TNFα that is not biologically active (Candon, et al., 2005; Charles, et al., 1999).

Little is known about the nature of the C-reactive protein in the rat. It is known, however, that the basal serum concentration of the protein in the rat is substantially higher than those in humans with no apparent toxicity. Nevertheless, our preliminary data indicated that pre-AA results in elevation of the already high CRP concentration (Figure 3-1 (Ling and Jamali, 2005) and 4-2 (Chapter 4)).

We, therefore, confirmed the presence of inflammation at the time of infliximab administration, by determining serum nitrite and C-reactive protein (CRP) concentrations.

Animal were inspected for signs and symptoms of adjuvant arthritis during the study period including joint swelling and deformity, development of nodules and signs of pain or distress including vocalizing, favoring of joints or immobility

(Whitehouse, 1988). Rats that developed any of these symptoms were euthanized and not used in experiments.

Rationale for dose and route of infliximab and verapamil administration

In humans, infliximab doses of 3 to 10 mg/kg are used intravenously. In the rat, subcutaneous doses of infliximab has been shown to have equal bioavailability and pharmacokinetic profile as iv doses (Yang, et al., 2003). The half life of elimination of infliximab is approximately 12 to 16 days in rats (Yang, et al., 2003). We therefore chose to administer single 10 mg/kg doses of infliximab subcutaneously as infliximab was expected to still be in circulation at the time we performed our experiments.

The suitability of single 10 mg/kg doses of infliximab was confirmed through a preliminary experiment (Chapter 4). The data generated indicated that in pre-AA rats with suppressed total microsomal cytochrome P450 content, the enzyme level was elevated 8 days after single 10 mg/kg doses of infliximab (Figure 5-2). Although infliximab may reduce inflammation within hours of dosing (Kreutz, et al., 2004; Kulmatycki, et al., 2001; Oruc, et al., 2004), hepatic enzyme recovery may require days, particularly if the down regulation was at the transcription level. Studies on the inducible expression of CYP's 1A1, 1A2 and 3A4 in human hepatocytes indicate that maximum induction is reached at 12 to 30 hours for mRNA and at least 48 hours for CYP proteins, in addition to protein half-life of 35 to 37 h (Muntane-Relat, et al., 1995; Shiraki and Guengerich, 1984). We, therefore, compared the microsomal content and verapamil pharmacokinetics in pre-AA rats following single 10 mg/kg doses of subcutaneous infliximab 8 days post-infliximab dose with those in placebo-treated healthy control rats.

Verapamil was administered by iv bolus injection as previously described (Ling and Jamali, 2005). Inflammation is expected to have a greater impact on the clearance of oral doses of highly extracted drugs as compared with those of iv doses. However, administration of the drug through the iv route is associated with smaller inter-animal variability than oral doses (Ling and Jamali, 2005). We have previously shown that pre-AA results in significant reduction in verapamil clearance even after iv administration (Ling and Jamali, 2005).

Protocol

For pharmacokinetic and microsomal experiments, animals were divided into 4 treatment groups according to the time course depicted in Figure 5-1: 1) Pre-adjuvant arthritis (pre-AA) rats were injected with adjuvant (0.2 mL of 50 mg/mL *Mycobacterium butyricum* suspended in squalene into the tail base) on day 0 and normal saline on day 6; 2) pre-AA/infliximab rats were injected with adjuvant on day 0 and infliximab (10 mg/kg by subcutaneous injection at a concentration of 10 mg/mL) on day 6; 3) control rats were administered normal saline (0.2 mL) on day 0 and day 6 and; 4) control/infliximab rats were administered saline on day 0 followed by infliximab (10 mg/kg) on day 6.

On day 13, the right jugular vein was cannulated for serial blood collection while animals were under halothane/O_2 anesthesia. A polyethylene (PE-50; Clay

Adams, Parsippany, NY) cannula tipped with 2 cm of silastic (Dow Corning Corp, Midland, MI) tubing was inserted into the right jugular vein and exteriorized by subcutaneous tunneling to an incision made in the interscapular area.

For the pharmacokinetic study, on day 14, one set of rats (n=8-10/group) received single doses of racemic verapamil intravenously (2 mg/kg in saline). Blood samples (~0.2 mL) were collected at 0, 0.08, 0.17, 0.25, 0.5, 1, 2, 4 and 8 h. Plasma was separated and kept at -80° C for verapamil assay.

A separate set of animals (n=8/group) were subjected to the protocol described above, but were not administered verapamil. Rats were sacrificed on day 14 and their livers removed and microsomes prepared (Barakat, et al., 2001). Briefly, livers were rinsed in 0.15 *M* KCl solution and homogenized in 0.25 *M* sucrose solution. The homogenates were centrifuged at 12000 *g* at 4° C for 10 min. The S9 fraction was collected and microsomes precipitated by addition of 1 *M* $CaCl_2$. The suspension was centrifuged at 27000 *g* at 4° C for 15 min, then the pellet was re-suspended in 0.15 *M* KCl solution and centrifuged at 27000 *g* at 4° C for 15 min. The pellet was again re-suspended in 0.25 *M* sucrose solution and stored at -80° C.

The microsomal protein concentration was determined by the Lowry method (Lowry, et al., 1951). Briefly, microsomes were incubated with 1% $CuSO_4$:2% Na.K.Tartarate:10% Na_2CO_3 anhydrous in 0.5 M NaOH (1:1:20 v/v/v) at room temperature for 10 min, then with 10% Folin-Phenol reagent at 50° C for 10 min. The sample was analyzed by spectrophotometry at 570 nm.

Total cytochrome P450 content was determined according to the method of Omura and Sato (Omura and Sato, 1964). Briefly, microsomes were suspended in 0.05 *M* phosphate buffer, pH 7.4, at a protein concentration of 1 mg/mL. Ten milligrams of solid sodium dithionite were added to the 1 mL of suspension and a baseline determined using the recording spectrophotometer by scanning from 500 to 400 nm. Carbon monoxide was bubbled gently into the sample cuvette for 20 sec. The spectrum was again recorded from 500 to 400 nm. The quantity of cytochrome P450 was calculated from the optical density difference (450-480 nm) and the molar extinction coefficient of 91 $mM^{-1}cm^{-1}$.

Western Blot Analysis

CYP3A and CYP1A protein content was assessed by Western Blot analysis. Microsomal protein (30 μg) was denatured at 100° C for 5 min, then separated by SDS-polyacrylamide gel electrophoresis on 10 % polyacrylamide gels, and blotted onto a pure nitrocellulose membrane (0.45 μm, Trans-Blot Transfer Medium, BioRad Laboratories, Hercules, CA). Nonspecific binding sites were blocked overnight in a solution of 5% skim milk:2% BSA:0.05% Tween-20 in TBS buffer. Membranes were incubated with primary antibody (1:1000 dilution; polyclonal rabbit anti-rat CYP3A2 or polyclonal goat anti-rat CYP1A1 (Daiichi Pure Chemicals, Tokyo, Japan)) for 2 h, and then with secondary antibody (1:5000 dilution; horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody or HRP-rabbit anti-goat IgG antibody, (Jackson Immunoresearch Laboratories Inc., West Grove, PA) for 1 h. Immunoreactive proteins were visualized by chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences, England) and band density measured by densitometry (TBX Densitometer, Tobias Associates Inc., Ivyland, PA).

Verapamil Protein Binding

Serum protein binding was measured in serum obtained from the set of rats used for microsomal experiments. One mL serum from each group of rats (n=7-8/group) were adjusted to pH 7.4 with 0.1 N HCl. The serum was spiked with 2000 ng/mL of racemic verapamil to approximate the serum concentrations of verapamil in rats after iv dosing. The serum was incubated at 37° C for 1 h, then transferred to Millipore micropartition chambers (Millipore Corporation, Bedford, MA) for ultrafiltration at 2000 *g* for 1 h. Filtrate and nonfiltrate verapamil concentrations were measured by HPLC. The fraction unbound, f_u, was determined as, $f_u=C_u/C_t$, where C_u is the unbound concentration and C_t is the total concentration.

Stereospecific Verapamil assay

A stereospecific high performance liquid chromatography (HPLC) method (Shibukawa and Wainer, 1992) was used to determine plasma concentrations of R and S verapamil. Briefly, 75 µl of (+)-glaucine (400 ng/mL) as internal standard was added to 100 µl plasma, followed by 100 µl of 2 *M* NaOH and 0.4 mL sodium phosphate buffer (pH 7.0, ionic strength 0.1). Verapamil was extracted with 6 mL heptane:heptafluorobutanol (99:1) and vortex mixed, followed by centrifugation. The organic layer was evaporated to dryness, the residue reconstituted in mobile phase (hexane-isopropanol-ethanol-TEA, 92:4:4:0.1v/v/v/v) and injected into an isocratic HPLC system at a flow rate of 0.7 mL/min. The HPLC apparatus consisted of a Waters WISP 712 autoinjector (Milipore-Waters, Mississauga, Canada), an achiral column (5 cm x 4.6 mm I.D. Supelcosil LCSi, Supelco Inc., Bellefonte, PA) and chiral column (250 mm x 4.6 mm I.D., 5 µm Chiralpak AD-H column, Daicel Chemical Inc., Tokyo, Japan) maintained at 31°C, a 474 fluorescence detector (Waters, Mississauga, Canada) set at excitation of 272 nm and emission at 317 nm with a bandwidth at 18 nm, and a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA). Standard curves were linear over the concentration range of 10 – 1000 ng/mL ($r^2 \geq 0.99$, cv $\leq 10\%$). The minimum quantifiable concentration was 5 ng/mL for S- and R-verapamil.

Serum Nitrite Analysis

Serum nitrite (NO_2^-), a stable breakdown product of nitric oxide (NO), was measured using a previously described method (Grisham, et al., 1996). Briefly, nitrate (NO_3^-) was reduced to nitrite (NO_2^-) by incubating 100 µl of serum with 10 µl of *Aspergillus* nitrate reductase (10 U/mL) in the presence of 25 µl 1 *M* HEPES (pH 7.4), 25 µl 0.1 m*M* FAD and 50 µl 1 m*M* NADPH for 30 min at 37° C. Then, 5 µl of lactate dehydrogenase (1500 U/mL) and 50 µl of 100 m*M* pyruvic acid were added and incubated for an additional 10 min at 37° C. NO_2^- was determined by addition of 1.0 mL Griess reagent and absorbance measured at 543 nm.

Standard curves were linear over the concentration range of 3 – 200 μ*M* ($r^2 \geq 0.99$, cv ≤10%). The minimum quantifiable concentration was 3 μ*M*.

Serum C-reactive Protein Analysis

A commercially available rat-CRP ELISA kit (Helica Biosystems, Inc., Fullerton, CA) was used. This assay required 100 μl of serum (1:10 000 dilution) to be added to a 96 well plate coated with antibodies to rat-CRP. After incubation for 30 min, the plate was washed and 100 μl of Conjugate (horseradish peroxidase (HRP)-labeled rabbit anti-rat CRP-IgG) was added and incubated for 30 min. The plate was again washed and 100 μl of TMB substrate solution was added and incubated for 10 min. Stop Solution (100 μl) was added to stop the reaction and absorbance was read at 450 nm. Standard curves were linear over the concentration range of 17.5 – 133 μg/mL ($r^2 \geq 0.99$, cv ≤10%). The limit of detection of the assay was 2.5 μg/mL.

Data Analysis and Statistics

Pharmacokinetic indices for S and R verapamil after iv administration were determined by noncompartmental analysis using WinNonlin 4.1 (Pharsight, Mountain View, CA).

Data is presented as mean ± standard deviation. Differences between treatment groups for pharmacokinetic parameters and CYP content were determined by analysis of variance with Tukey's adjustment for multiple comparisons. Differences in plasma concentrations and inter-group and inter-day differences in animal weight, serum nitrite and CRP concentrations were determined by analysis of variance for repeated measures with Tukey's adjustment for multiple comparisons. Statistical significance was set at $p \leq 0.05$.

Results

Pre-AA and pre-AA/infliximab rats gained significantly less weight than control rats (Figure 5-3). Of 38 rats that were injected with adjuvant, 6 developed visible symptoms of arthritic disease, including swelling of one or both hind limbs, and immobility of the affected limb, during the 14 day experimental period and were euthanized upon appearance of symptoms. Only asymptomatic animals were used in experiments.

Serum nitrite concentrations were significantly elevated in pre-AA rats as compared with control rats on day 6 and day 14 (Figure 5-4). Treatment with infliximab (pre-AA/infliximab) had no significant effect on the elevated plasma nitrite concentrations on day 14 compared with pre-AA rats not treated with infliximab. Infliximab also had no significant effects on nitrite concentrations in control rats (Figure 5-4). There was a trend toward increased C-reactive protein concentrations in pre-AA rats that did not reach statistical significance ($p<0.06$). Infliximab did not significantly influence CRP concentrations as, on day 14, no significant differences were found among the four groups (Figure 5-4).

As expected, pre-AA resulted in increased plasma concentrations of S-verapamil (Table 5-1, Figure 5-5). In terms of AUC, the increase was 72% for the pre-AA and 96% for the pre-AA/infliximab rats as compared with their respective controls. Due to the variability in data, however, only the difference in pre-AA/infliximab reached significance (p=0.08, pre-AA; p=0.006, pre-AA/infliximab). Increases in S-verapamil AUC, however, were significantly correlated with increases in serum nitrite and CRP (Figure 5-6). A corresponding decrease in clearance was observed (35% for pre-AA, p=0.10; 38% for pre-AA/infliximab, p=0.04) that was also significant for the pre-AA/infliximab group compared with controls. Infliximab treatment did not have any significant effects on plasma concentrations or pharmacokinetic indices in healthy rats.

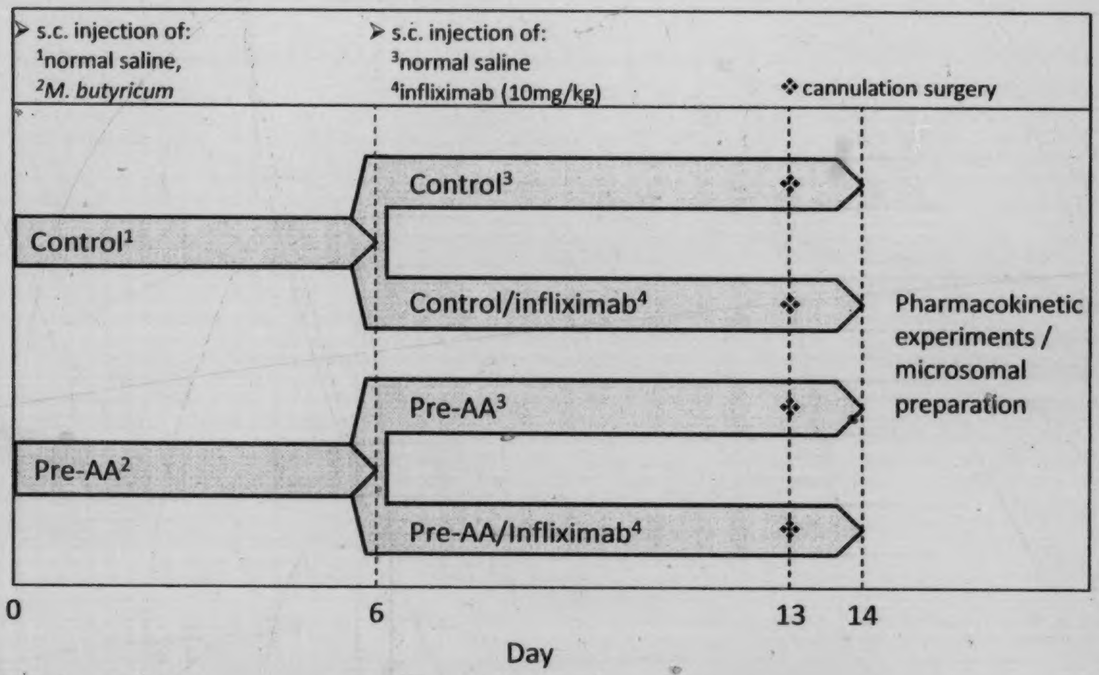
Protein binding of verapamil was significantly increased by pre-AA (Table 5-1). Unbound fraction was reduced by 74% for S- and 53% for R-verapamil compared with controls. Infliximab did not alter verapamil protein binding with respect to either control or inflamed animals. Plasma concentrations and pharmacokinetic indices of R-verapamil were not significantly different among the four groups of rats.

Total microsomal cytochrome P450 content was significantly reduced in pre-AA rats compared with control rats (p<0.001) (Figure 5-7). Microsomal content of CYP3A1/2 and CYP1A1/2 isoforms were also significantly down-regulated in pre-AA rats (Figure 5-7) and the reduction was correlated with the reduction in total P450 (Figure 5-8). Infliximab treatment in pre-AA rats resulted in significantly higher cytochrome P450 and CYP3A1/2 protein content relative to pre-AA rats, although levels of both proteins remained significantly reduced compared with controls (Figure 5-8). CYP1A1/2 isoenzyme content was not significantly different from controls in infliximab treated pre-AA rats. Infliximab did not significantly alter total P450 or CYP isoenzyme content in healthy rats. The reductions in total P450 content as well as suppression of both CYP3A1/2 and CYP1A1/2 protein expression were significantly correlated with increases in serum nitrite but not CRP levels (Figure 5-9).

TABLE 5-1. Pharmacokinetic indices of verapamil following single intravenous 2 mg/kg doses of racemic verapamil (n=7-10/group).

	Control	Control/Infliximab	Pre-AA	Pre-AA/Infliximab
$t_{1/2}$(h)				
S	3.8 ± 1.5	4.3 ± 1.6	3.4 ± 1.0	2.4 ± 2.1
R	2.4 ± 0.8	2.2 ± 0.6	2.6 ± 1.3	1.9 ± 0.8
AUC_{0-8} (μg.min/mL)				
S	36.2 ± 7.3	36.7 ± 7.0	61.8 ± 22.7	70.4 ± 25.6**
R	33.8 ± 8.4	34.5 ± 5.6	39.3 ± 6.4	41.9 ± 13.1
$AUC_{0-\infty}$ (μg.min/mL)				
S	39.9 ± 8.8	40.8 ± 8.7	64.7 ± 24.0	72.7 ± 26.8**
R	35.9 ± 10.1	36.1 ± 6.3	41.1 ± 8.1	43.1 ± 14.0
CL (L/min/kg)				
S	0.026 ± 0.007	0.025 ± 0.005	0.017 ± 0.007	0.016 ± 0.008*
R	0.030 ± 0.009	0.028 ± 0.005	0.025 ± 0.005	0.026 ± 0.009
Vz (L/kg)				
S	8.5 ± 2.9	9.4 ± 4.2	5.0 ± 2.6	3.0 ± 2.8**
R	5.8 ± 1.4	5.4 ± 1.6	5.4 ± 2.2	3.9 ± 1.2
Vss (L/kg)				
S	3.7 ± 1.1	3.8 ± 1.3	1.6 ± 0.6***	1.2 ± 0.7***
R	3.5 ± 0.7	3.2 ± 0.4	2.3 ± 0.6**	2.0 ± 0.7***
f_u				
S	0.070 ± 0.024	0.061 ± 0.019	0.018 ± 0.009***	0.021 ± 0.009***
R	0.120 ± 0.033	0.111 ± 0.028	0.057 ± 0.031***	0.068 ± 0.022**

*p<0.05, **p<0.01, ***p<0.001, significantly different from control



➢ s.c. injection of:
¹normal saline,
²M. butyricum
➢ s.c. injection of:
³normal saline
⁴infliximab (10mg/kg)
❖ cannulation surgery
Control¹
Control³
Control/infliximab⁴
Pre-AA²
Pre-AA³
Pre-AA/Infliximab⁴
Pharmacokinetic experiments / microsomal preparation
0
6
13
14
Day

FIGURE 5-1. Time course of experimental protocol. All rats received adjuvant (*M. butyricum*) or normal saline on day 0. On day 6, Control/infliximab and Pre-AA/infliximab groups received 10 mg/kg doses of infliximab by sc injection. Control and Pre-AA rats received normal saline. On day 13, all rats were cannulated in the right jugular vein. On day 14, rats were used for pharmacokinetic experiments (administered verapamil followed by serial blood collection for 8 h) or sacrificed and livers removed for microsome preparation.

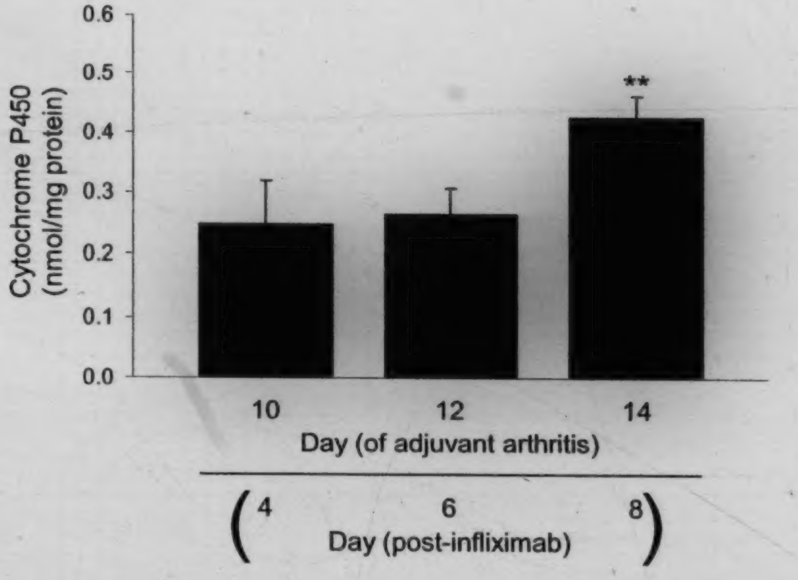


FIGURE 5-2. Mean total cytochrome P450 content 4, 6 and 8 days following a single 10 mg/kg dose of infliximab in pre-AA/infliximab rats (n=3/group). Error bars represent standard deviation of the means. **$p \leq 0.01$ (ANOVA).

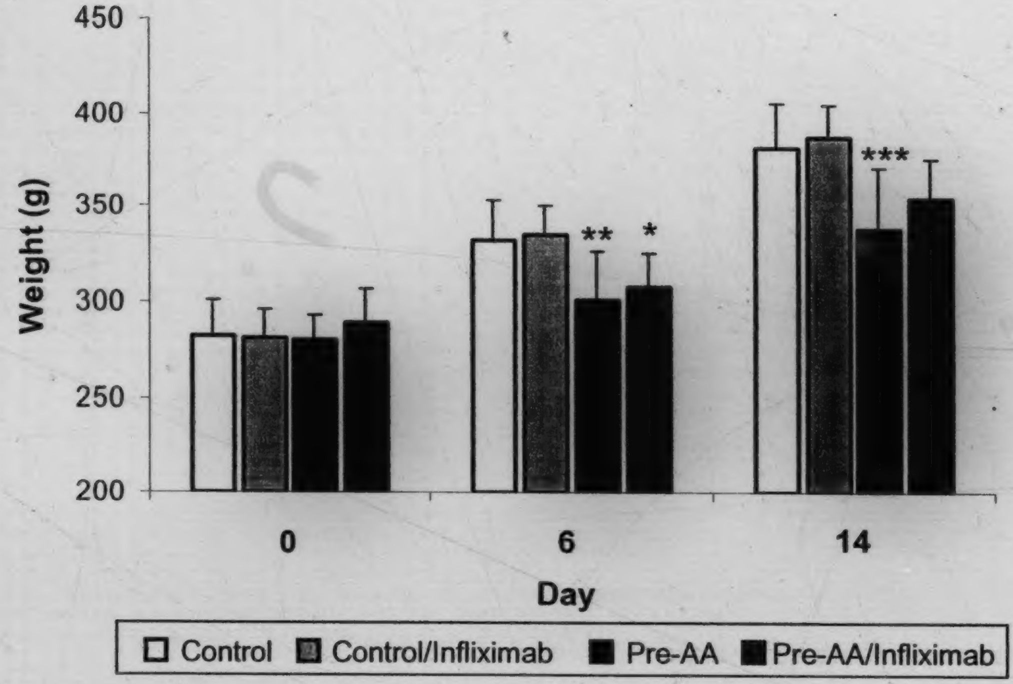


FIGURE 5-3. Mean animal body weight at baseline (day 0), on the day of infliximab dosing (day 6) and 8 days post-infliximab (day 14). Error bars represent standard deviation of the means. Significance of difference from control: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

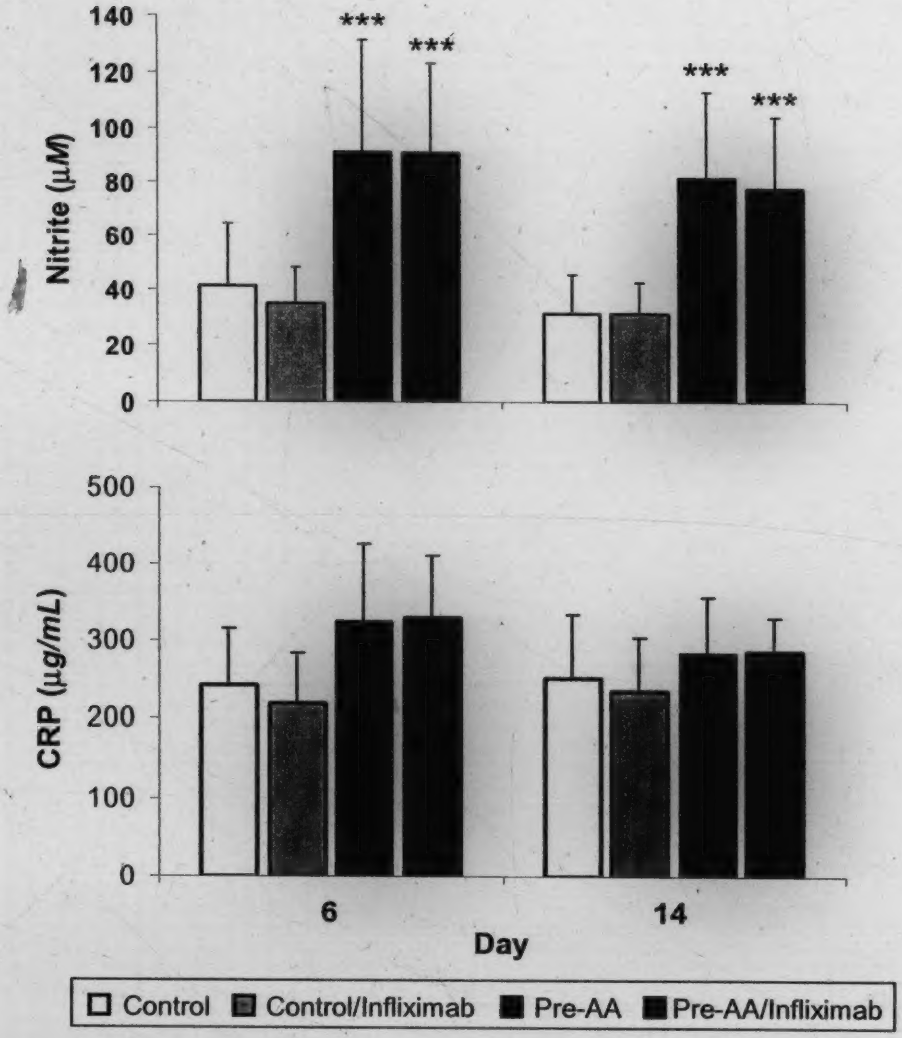


FIGURE 5-4. Mean serum nitrite and C-reactive protein concentrations on day of infliximab dosing (day 6) and 8 days post-infliximab (day 14). Error bars represent standard deviation of the means. Significance of difference from control: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

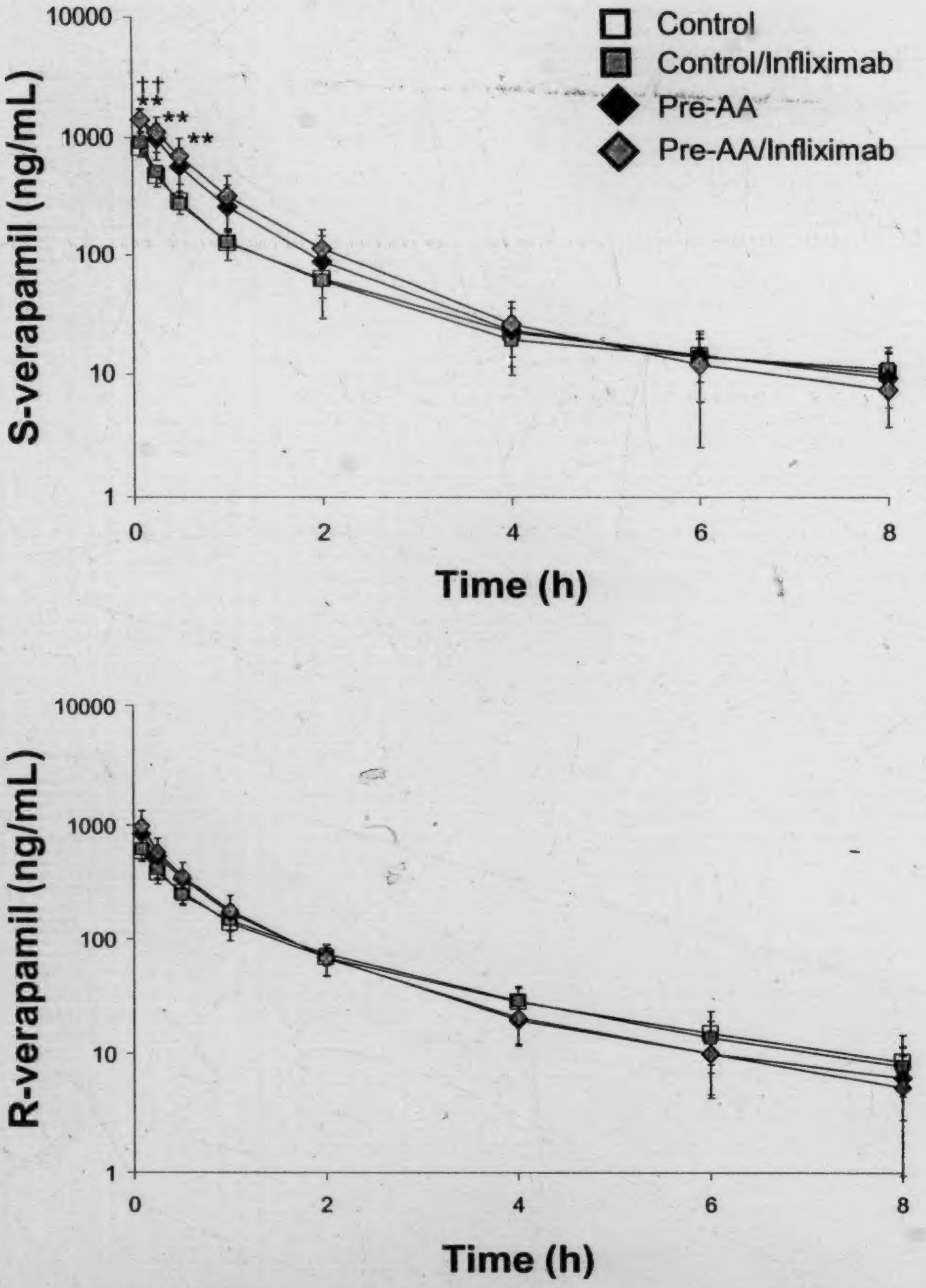


FIGURE 5-5. Mean plasma verapamil concentration versus time curves at day 14 (8 days post-infliximab) following administration of single intravenous 2 mg/kg racemic verapamil doses. Error bars represent standard deviation of the means. Lines connect experimental data points. Significance of difference from control: $^{††}p<0.01$ (Pre-AA). $^{**}p \leq 0.01$ (Pre-AA/Infliximab).

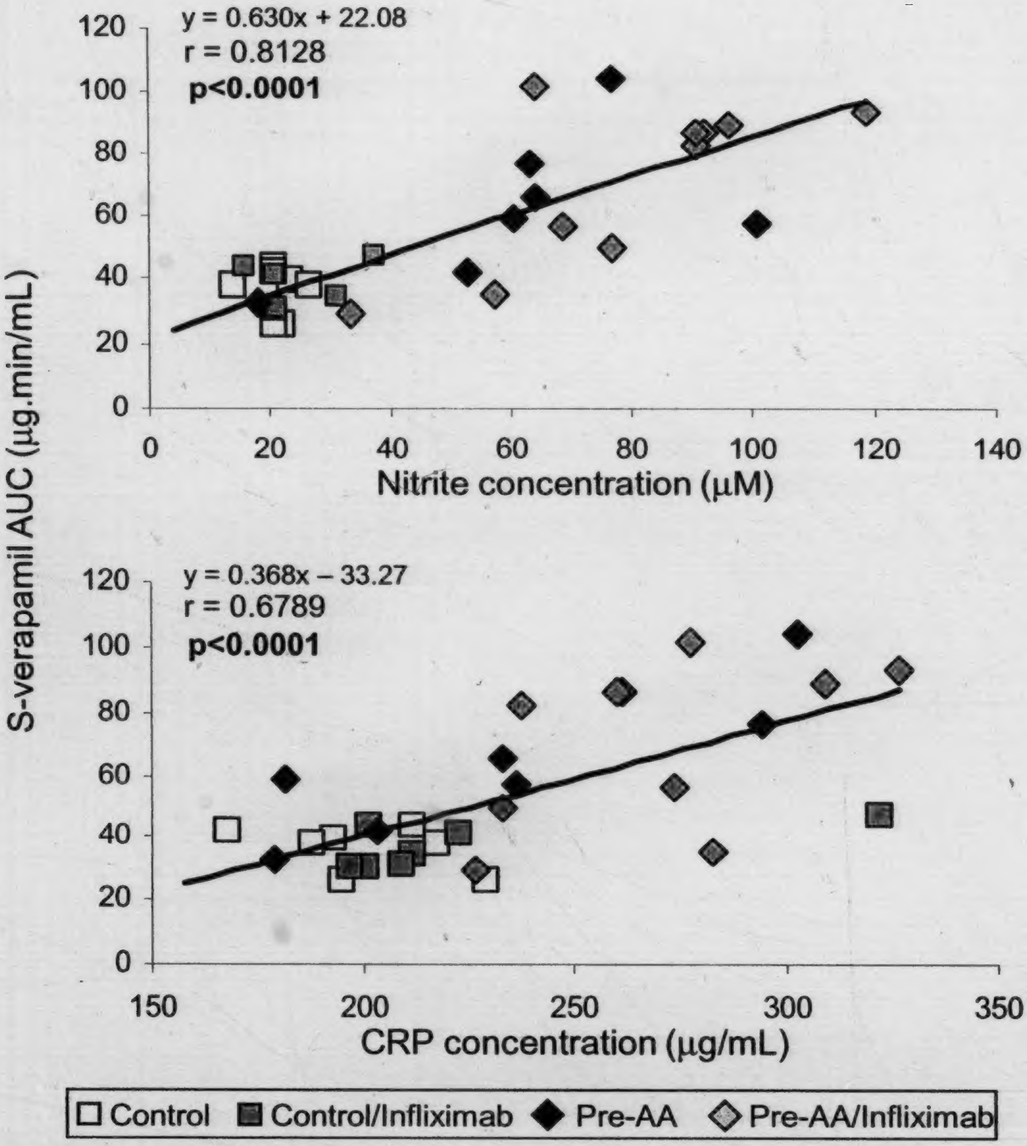


FIGURE 5-6. The correlation between S-verapamil area under the plasma concentration-time curve and serum nitrite and C-reactive protein concentrations. Regression lines are depicted.

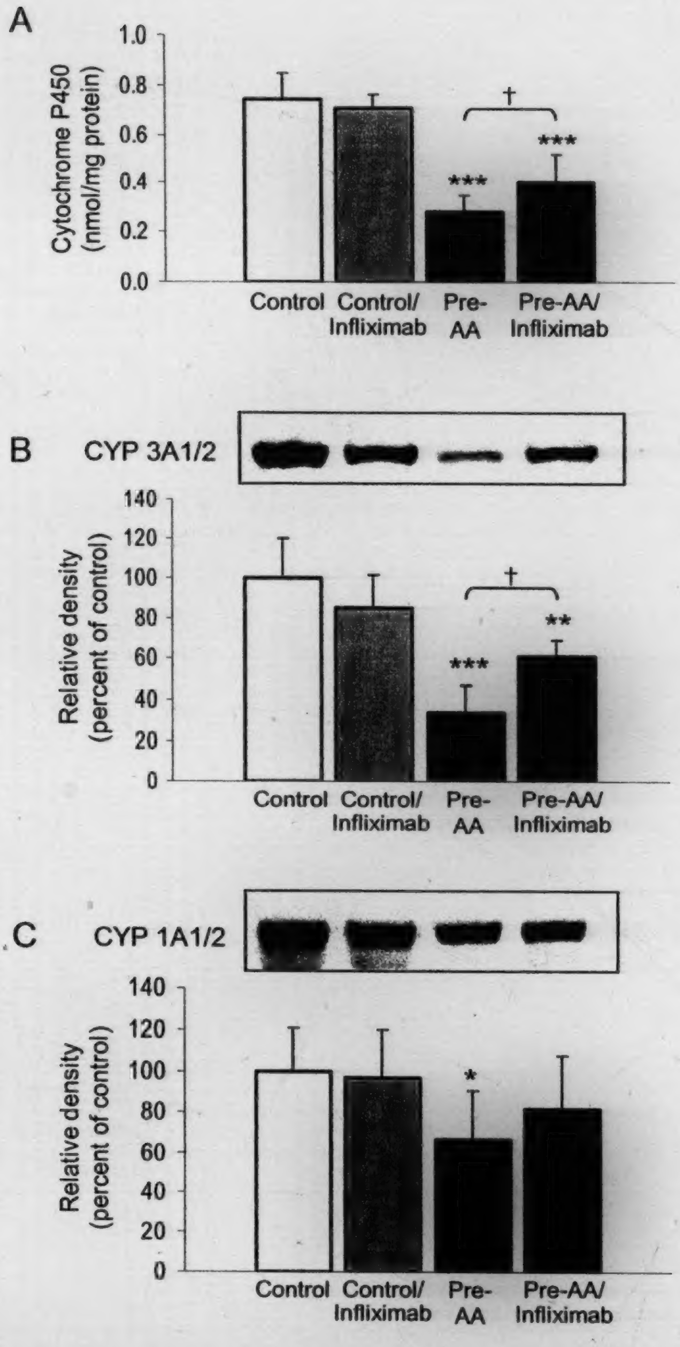


FIGURE 5-7. (A) Mean total cytochrome P450 content; (B) CYP3A1/2 Western blot and relative protein density; (C) CYP1A1/2 Western blot and relative protein density. Error bars represent standard deviation of the means.: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, significantly different from control; $^{\dagger}p<0.05$, significant difference between groups.

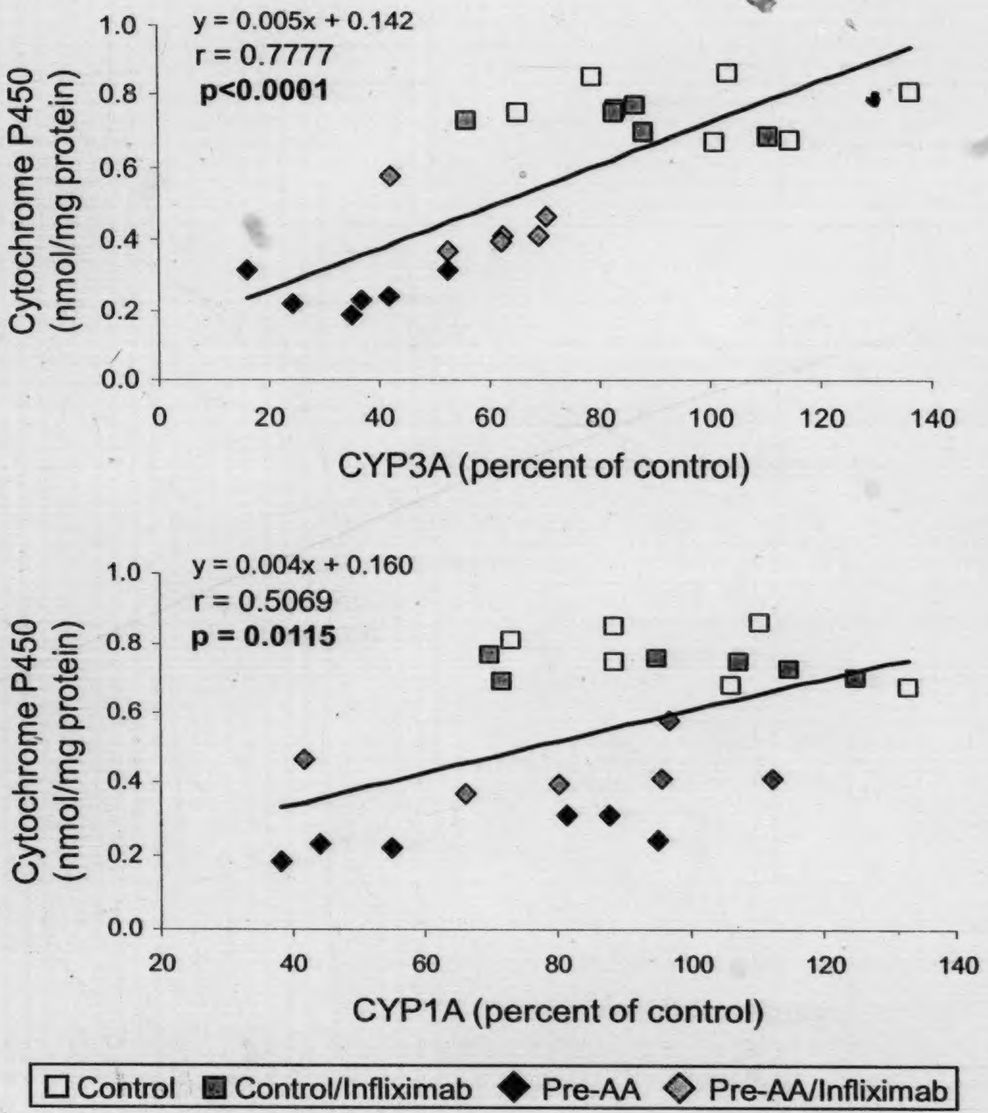


FIGURE 5-8. The correlation between total cytochrome P450 content and CYP3A and CYP1A protein expression. Regression lines are depicted.

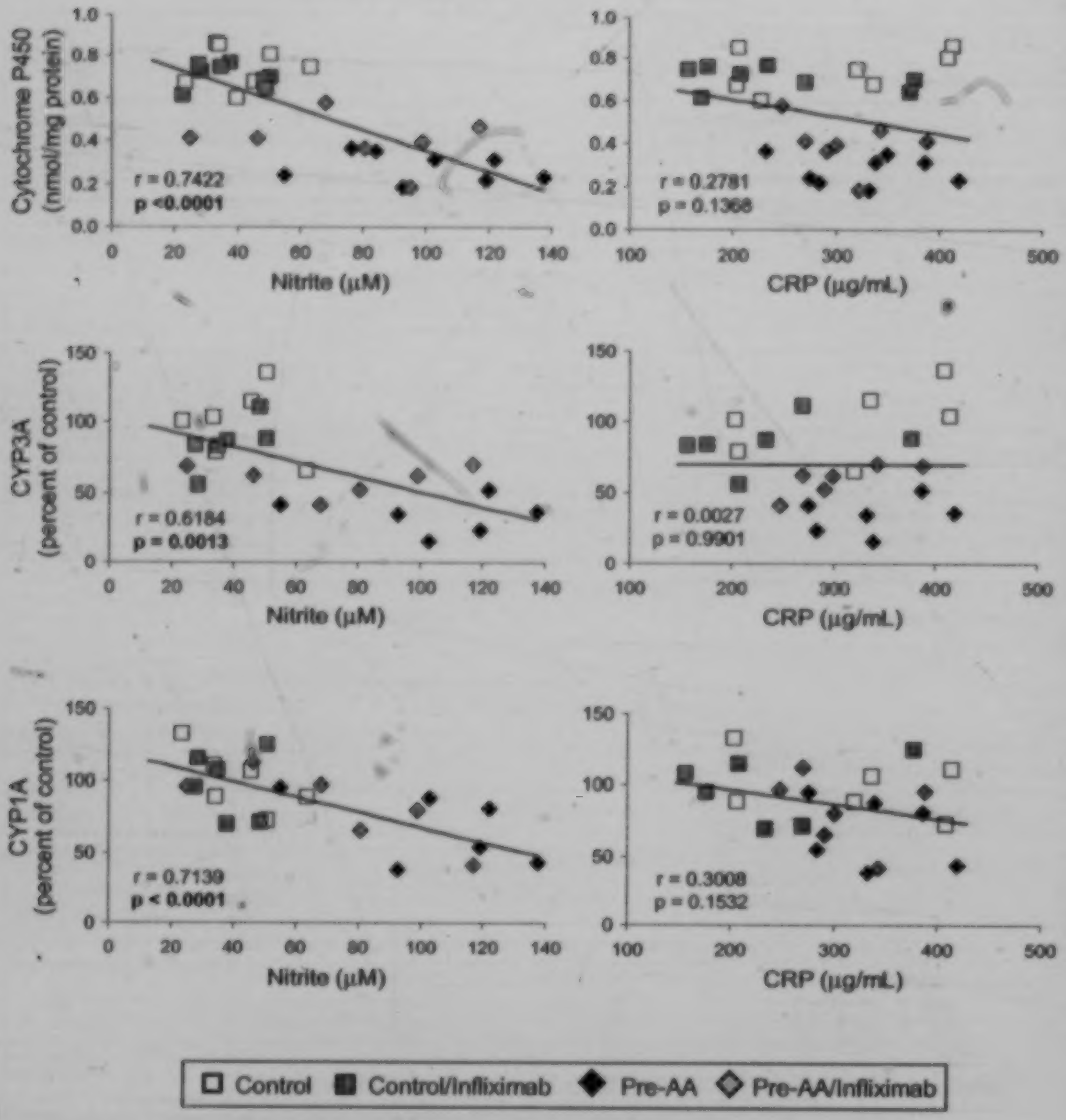


FIGURE 5-9. The correlation between total cytochrome P450 content, CYP3A and CYP1A protein expression and serum nitrite and C-reactive protein concentrations. Regression lines are depicted.

Discussion

As expected (Ling and Jamali, 2005), pre-AA was associated with increased serum nitrite concentrations (Figure 5-4), reduced rate of body weight gain (Figure 5-3) and diminished total hepatic cytochrome 450 enzymes (Figure 5-7) and elevated plasma verapamil concentrations (Figure 5-5, Table 5-1). The present data indicate that the diminished total CYP450 appear to be due to a decreased expression of CYP proteins and is associated with diminished contents of isozymes involved in clearing verapamil, i.e., CYP3A and CYP1A (Figure 5-7, Table 5-2). Indeed, strong positive correlations were observed between the total CYP content and CYP3A and CYP1A (Figure 5-8). This, at least in part, may explain the observed reduced verapamil clearance following oral and iv doses (Ling and Jamali, 2005) since these enzymes are involved in the clearance of verapamil (Kroemer, et al., 1993). The observed increased binding of verapamil to plasma proteins (Table 5-1) may also contribute to the reduced drug clearance in pre-AA.

Interestingly, the microsomal content of the examined isozymes were positively correlated with the circulating nitrite concentrations (Figure 5-9) and, in turn, S-verapamil AUC correlates well with the nitrite concentrations (Figure 5-6). A similar association, i.e., between nitrite and verapamil clearance has also been shown in human rheumatoid arthritis (Mayo, et al., 2000). Correlation between R-verapamil AUC and serum nitrite and CRP was non-significant.

Treatment with infliximab resulted in significantly increased cytochrome P450 and CYP3A protein content as compared with pre-AA rats, although these concentrations were still significantly lower than in controls (Figure 5-7). The increases in CYP isozyme, however, did not result in normalization of S-verapamil clearance (Figure 5-5, Table 5-1). In general, systemic clearance of drugs that are efficiently cleared in the liver, i.e., the high hepatic extraction ratio drugs, is dependent primarily on hepatic blood flow (Q) (Gibaldi and Perrier, 1982). In contrast, oral clearance of high extraction efficiency drugs is dependent also on hepatic enzyme function as these drugs undergo extensive first pass extraction. Pre-AA would therefore be expected to have a greater effect on verapamil pharmacokinetics after oral rather than iv administration since pre-AA suppresses CYP enzyme concentration but not hepatic blood flow (Walker, et al., 1986). Under inflammatory conditions, however, metabolic activity may be suppressed to such an extent that extraction efficiency is reduced to that of intermediately extracted drugs and consequently, intrinsic clearance becomes dependent on drug free fraction and intrinsic metabolic activity in addition to Q (Gibaldi and Perrier, 1982; Ling and Jamali, 2005). In the present study, the hepatic extraction decreased from 0.86 to 0.56 for S-verapamil in pre-AA rats; values consistent with a change in extraction ratio from a highly cleared to intermediately cleared drug. Such a substantial reduction in intrinsic metabolic activity was expected (Ling and Jamali, 2005) and consistent with the observed reduction in CYP protein content (Figure 5-7). Clearance of drugs with intermediate hepatic extraction ratio depends on their plasma protein binding. Infliximab did not normalize plasma protein binding of verapamil (Table 5-1), hence, the anti-TNF treatment failed to normalize pharmacokinetics of the drug in pre-AA despite increased enzyme expression. The reason for the infliximab failure to influence verapamil plasma protein binding remains unknown.

Interestingly, however, infliximab treatment did not significantly reduce the elevated serum nitrite levels on day 14 (Figure 5-4). This was surprising since infliximab as single or multiple doses has been shown to reduce serum nitrite concentrations in acute inflammation (Kulmatycki, et al., 2001), reduce NOS2 expression in peripheral blood mononuclear cells of RA patients (Perkins, et al., 1998) and NO production in cartilage of osteoarthritis patients (Vuolteenaho, et al., 2002). Similarly, in the rat, anti-rat-TNFα antibody has been shown to reduce serum levels of nitrite and iNOS expression in indomethacin-induced enteropathy (Saud, et al., 2005). The discrepancy may be due to the fact that although the rat specific anti-TNFα antibodies has been shown to decrease inflammation (Bush, et al., 2002; Kreutz, et al., 2004; Murata, et al., 2004; Murata, et al., 2005; Olmarker, et al., 2003; Onda, et al., 2004; Oruc, et al., 2004; Williams, et al., 2000; Woodruff, et al., 2003), the present work reports, to the best of our knowledge, for the first time, the effect of infliximab, a chimeric mouse/human monoclonal antibody, on experimental arthritis. The elevated levels of serum nitrite in pre-AA might have been caused by a cytokine-mediated T cell function, which has been shown not to be suppressed by TNFα blockade in early phase of AA (Bush, et al., 2002).

Although we have previously shown elevated CRP concentrations in pre-AA (Ling and Jamali, 2005), in this study we noticed only an insignificant trend (Figure 5-4). This might have been due to variability in the disease activity among the pre-AA group. In addition, in the rat, CRP is present in much greater concentrations than in humans, where levels are increased several fold in RA (Familian, et al., 2005), while in the rat, the increase in less than 2 fold (Ling and Jamali, 2005). Indeed, in human, CRP concentrations several fold lower than in rats results in high mortality rate (Pietila, et al., 1996; Verheggen, et al., 1999). This may indicate inter-species difference in either the structure or toxicity of the protein. Nevertheless, CRP appears to be not as reliable a marker of arthritis as nitrite. In humans also, nitrite has been shown to be a reliable predictor of RA (Mayo, et al., 2000).

It is notable that regardless of the treatment, both CYP1A and 3A isoenzyme levels were significantly correlated with serum nitrite concentrations (Figure 5-9), suggestive of a prominent role of NO in CYP down-regulation in pre-AA. Nitric oxide has been shown to inactivate cytochrome P450 enzymes (Khatsenko, et al., 1993) and perhaps mediates cytokine-induced suppression of CYP450 activity and content (Morgan, 1997).

The purpose of this study was to determine the effect of infliximab on hepatic CYP content and the pharmacokinetics of verapamil in pre-arthritic (pre-AA) rats. We found infliximab treatment to be associated with significant increases in total microsomal CYP450 and CYP3A isoenzymes. Furthermore, decreased CYP enzymes expressions were significantly correlated with serum nitrite but not CRP levels, suggesting a prominent role of NO in CYP down-regulation. Despite limited recovery of CYP enzymes following infliximab treatment, however, verapamil clearance remained inhibited in pre-AA rats, due perhaps, to a lack of effect of infliximab on verapamil plasma protein binding.

CHAPTER 6

Effect of infliximab on verapamil pharmacokinetics and pharmacodynamics in rheumatoid arthritis

Introduction

Inflammation reduces clearance of some cardiovascular drugs including the beta-adrenergic blocker propranolol and the calcium channel antagonist verapamil, while paradoxically reduces pharmacodynamic response (Guirguis and Jamali, 2003; Mayo, et al., 2000; Sattari, et al., 2003). Reduced responsiveness to cardiovascular drugs may contribute to the increased risk of cardiovascular morbidity and mortality observed in patients with rheumatoid arthritis (RA) in addition to the pathogenic role of inflammatory mediators on atherosclerotic development (Maradit-Kremers, et al., 2005a; Maradit-Kremers, et al., 2005b; McEntegart, et al., 2001; Myllykangas-Luosujarvi, et al., 1995; Pasceri and Yeh, 1999; Sattar, et al., 2003; Solomon, et al., 2003; Wolfe, et al., 1994).

Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), and IL-6 are found in the inflamed joints and sera of RA patients (Feldmann and Maini, 1999) and may be involved in joint destruction and inflammation. Elevated systemic levels of pro-inflammatory cytokines can also alter metabolic functions that lead to insulin resistance, dyslipidemia and endothelial dysfunction – all of which are pro-atherogenic risk factors (Sattar, et al., 2003). In addition, cardiovascular conditions including atherosclerosis, myocardial infarction, heart failure, and hypertension are associated with increased pro-inflammatory mediators (Cesari, et al., 2003; Peeters, et al., 2001; Ridker, et al., 2000) and are the predominant cause of mortality in patients with RA (Myllykangas-Luosujarvi, et al., 1995; Wolfe, et al., 1994).

Elevated pro-inflammatory cytokines in RA (Mayo, et al., 2000) and in various animal models of inflammation (Ling and Jamali, 2005; Sattari, et al., 2003) are also associated with suppression of hepatic metabolic processes (Kulmatycki, et al., 2001; Ling and Jamali, 2005; Morgan, 1997; Piquette-Miller and Jamali, 1993) and increased concentrations of acute phase proteins such as α_1-acid glycoprotein (AAG) (Belpaire, et al., 1982), resulting in reduced clearance and significantly elevated plasma drug concentrations of some cardiovascular drugs (Kulmatycki and Jamali, 2005).

The expected therapeutic consequence of reduced drug clearance is increased potency and toxicity. Under inflammatory conditions, however, suboptimal therapy or even therapeutic failure may result, as observed for cardiovascular drugs (Kulmatycki and Jamali, 2005). For example, pharmacologic response to verapamil is unexpectedly reduced in patients with active RA despite higher than normal drug concentrations (Mayo, et al., 2000). Since the unbound concentrations of the pharmacologically active S enantiomer of the drug are unchanged, the reduced response may be the result of down regulation of cardiac receptors, as has been observed in inflammation in the rat (Sattari, et al., 2003). Decreased drug response in the presence of inflammation in the rat has also been reported for the beta-adrenergic antagonists, propranolol (Guirguis and Jamali,

2003) and sotalol. The reduced pharmacodynamic response to sotalol is, however, normalized by the anti-TNFα drug, infliximab (Kulmatycki, et al., 2001).

Infliximab is a chimeric anti-TNFα antibody which binds and inactivates TNFα, resulting in reduced systemic levels of pro-inflammatory mediators and hence, improved clinical outcome in RA and Crohn's disease. The role of anti-TNF agents in cardiovascular disease is less clear. Although TNFα contributes prominently to heart failure, anti-TNF therapy has been associated with negative outcomes in patients with congestive heart failure (Chung, et al., 2003; Mann, et al., 2004). In spite of this, there is evidence that the risk of developing cardiovascular disease is lower in patients with RA treated with TNF blockers (Jacobsson, et al., 2005).

The purpose of this study was to determine if remission from RA, induced either by infliximab treatment or by other anti-rheumatic treatment is associated with a reversal of inflammation-induced changes in verapamil pharmacokinetics (PK) and pharmacodynamics (PD).

Materials and Methods

Chemicals

Verapamil 80 mg tablets were purchased from the University of Alberta Hospital Pharmacy (Edmonton, AB, Canada). Verapamil hydrochloride, (+) glaucine, heptafluorobutanol, a*spergillus* nitrate reductase (10 U mL^{-1}), HEPES, FAD, NADPH, lactic dehydrogenase (1500 U mL^{-1}), pyruvic acid, sulfanilamide, and naphthylethylenediamine dihydrochloride were purchased from Sigma

Chemical Co. (St. Louis, MO). High-performance liquid chromatography (HPLC) grade hexane and HPLC grade isopropanol, triethylamine (TEA), and 98% ethanol were purchased from Caledon Laboratories (Georgetown, Canada). Endogen Human TNFα ELISA kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL).

Subjects and Study Protocol

The protocol was approved by the University of Alberta Health Research Ethics Board and the study was performed in accordance with the declaration of Helsinki. Subjects were rheumatoid arthritis patients recruited from rheumatology clinics in the city of Edmonton, Alberta. All participants provided written informed consent. A total of 32 patients within the age range of 20 to 75 years were entered into the study. Included were 20 RA patients in remission; 12 of whom were undertaking infliximab therapy for at least 2 years and 8 who were on other non-biologic anti-rheumatic therapy; and 12 healthy volunteers. All subjects underwent physical and laboratory examinations within 1 month of study commencement. The screening studies consisted of complete blood counts, alanine aminotransferase (ALT) and serum creatinine as well as electrocardiogram (ECG) to ensure that there were no underlying cardiac, hematological, hepatic or renal abnormalities. Patients were allowed to be on anti-hypertensive medications including diuretics, ACE inhibitors, angiotensin receptor blockers and second-generation calcium channel blockers, however, beta blockers were to be excluded due to their potential to interfere with the pharmacodynamic response of the study medication as well as medications with potential pharmacokinetic interactions with verapamil. Non-antiarthritic medications were discontinued at least 24 h prior to verapamil administration and throughout the experiment.

All subjects fasted overnight prior to the study. On the study day, subjects were admitted to the University of Alberta Hospital Clinical Investigation Unit at 0700 h. After a 30 minute rest period, baseline physiological variables (heart rate, PR interval and blood pressure) were measured (Pulsewave, ECG). An intravenous line was established in the antecubital vein for serial blood sampling. At time 0 h, 80 mg of *rac*-verapamil (Apo-verapamil 80 mg) was administered with 200 mL water. Serial blood samples were drawn at 0, 30 min, and 1, 1.5, 3, 4, 6, 8 and 12 h. Serum and plasma were separated and stored at -70° C until analyzed. Blood pressure and ECG measurements were recorded 1 min prior to each blood sample. Serum nitrite, IL-6, TNFα and C-reactive protein (CRP) concentrations were measured in the time zero blood samples. Plasma verapamil enantiomers concentrations were analyzed by stereospecific HPLC assay. Subjects ate a standard breakfast and lunch after the 1.5 and 6 h samples, respectively.

Disease severity was clinically assessed according to the American Rheumatism Association 1987 revised criteria (Klippel, et al., 1997). An arthritis index was calculated using the number of joints involved and the severity as follows: 0, no joints involved; 1, 1-4 joints; 2, 5-9 joints; 3, >10 joints involved.

Swelling was assessed as 0, none; 1, mild; 2, moderate; 3, severe. A maximum score of 6 would indicate severe disease.

Electrocardiogram and Haemodynamic analysis

ECG was recorded using a standard lead I and aV5 electrocardiogram (Pulsewave, ECG). Systolic and diastolic blood pressure and mean arterial pressure was also recorded (Pulsewave, ECG). Three PR interval and heart rate measurements were recorded during the minute before blood samples collection for the pharmacokinetic analysis.

Stereospecific Verapamil assay

A stereospecific high performance liquid chromatography (HPLC) method (Shibukawa and Wainer, 1992) was used to determine plasma concentrations of R and S verapamil. Briefly, 75 µl of (+)-glaucine (400 ng/mL) as internal standard was added to 100 µl plasma, followed by 100 µl of 2 *M* NaOH and 0.4 mL sodium phosphate buffer (pH 7.0, ionic strength 0.1). Verapamil was extracted with 6 mL heptane:heptafluorobutanol (99:1) and vortex mixed, followed by centrifugation. The organic layer was evaporated to dryness, the residue reconstituted in mobile phase (hexane-isopropanol-ethanol-TEA, 92:4:4:0.1v/v/v/v) and injected into an isocratic HPLC system at a flow rate of 0.7 mL/min. The HPLC apparatus consisted of a Waters WISP 712 autoinjector (Milipore-Waters, Mississauga, Canada), an achiral column (5 cm x 4.6 mm I.D.

Supelcosil LCSi, Supelco Inc., Bellefonte, PA) and chiral column (250 mm x 4.6 mm I.D., 5 µm Chiralpak AD-H column, Daicel Chemical Inc., Tokyo, Japan) maintained at 31°C, a 474 fluorescence detector (Waters, Mississauga, Canada) set at excitation of 272 nm and emission at 317 nm with a bandwidth at 18 nm, and a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA). Standard curves were linear over the concentration range of 10 – 1000 ng/mL ($r^2 \geq 0.99$, cv $\leq$10%). The minimum quantifiable concentration was 5 ng/mL for S- and R-verapamil.

Verapamil Protein Binding

Protein binding was measured in plasma from RA (n=12) and healthy subjects (n=12). One mL of plasma was adjusted to pH 7.4 with 0.1 N HCl, and then spiked with 200 ng/mL of racemic verapamil to approximate the plasma concentrations of verapamil after oral dosing. The plasma was incubated at 37° C for 1 h, then transferred to Millipore micropartition chambers (Millipore Corporation, Bedford, MA) for ultrafiltration at 2000 *g* for 1 h. Filtrate and nonfiltrate verapamil concentrations were measured by HPLC. The fraction unbound, f_u, was determined as, $f_u = C_u/C_t$, where C_u is the unbound concentration and C_t is the total concentration.

Serum Nitrite Analysis

Serum nitrite (NO_2^-), a stable breakdown product of nitric oxide (NO), was measured using a previously described method (Grisham, et al., 1996). Briefly, nitrate (NO_3^-) was reduced to nitrite (NO_2^-) by incubating 100 μl of serum with 10 μl of *Aspergillus* nitrate reductase (10 U/mL) in the presence of 25 μl 1 *M* HEPES (pH 7.4), 25 μl 0.1 m*M* FAD and 50 μl 1 m*M* NADPH for 30 min at 37° C. Then, 5 μl of lactate dehydrogenase (1500 U/mL) and 50 μl of 100 m*M* pyruvic acid were added and incubated for an additional 10 min at 37° C. NO_2^- was determined by addition of 1.0 mL Griess reagent and absorbance measured at 543 nm. Standard curves were linear over the concentration range of 3 – 200 μ*M* ($r^2 \geq 0.99$, cv ≤10%). The minimum quantifiable concentration was 3 μ*M*.

Plasma TNFα Analysis

Plasma TNFα concentrations were measured by ELISA (Endogen Human TNFα ELISA kit, Pierce Biotechnology Inc., Rockford, IL). The sensitivity of the assay was 2 pg/mL (CV<10%).

Interleukin 6 assay

Serum interleukin (IL)-6 concentrations were measured by ELISA (Medgenix Diagnostics, Fleurus, Belgium). This allowed for the quantification of IL-6 to 2.0 pg/mL (CV < 8%).

Serum C-reactive Protein Analysis

CRP was analyzed by Dynacare Kasper Medical Laboratories (Edmonton, Canada).

Data Analysis and Statistics

Plasma S- and R-verapamil concentrations were analyzed by non-compartmental analysis using WinNonlin 4.1 (Pharsight, Mountain View, CA). Elimination rate constants (β) were calculated using log-linear regression of at least three points in the log-linear terminal phase of the plasma concentration-time curve. The area under the plasma concentration-time curve ($AUC_{0-\infty}$) was calculated using the log-linear trapezoidal rule until the last experimental data point (C_{last}) plus C_{last}/λ_z. AUC of the unbound fraction was calculated from $AUCu_{(0,\infty)} = AUC_{(0,\infty)} \times fu$, where *fu* is the fraction unbound and it is assumed that verapamil protein binding is concentration independent. Oral clearance (CL/*F*) was estimated for each patient from

$$CL/F = Dose/(AUC_{0-\infty})$$

Changes in PR interval, heart rate, systolic and diastolic blood pressure and mean arterial pressure were measured as responses to verapamil. Percent changes were calculated from the differences observed between the baseline and post-treatment values. The area under the percent effect-time curve was calculated using the trapezoidal rule.

The relationship between the PR interval and plasma S-verapamil concentrations was analyzed by PK/PD modeling using WinNonlin. Mean S-verapamil concentration and percent change in PR interval data were fitted to a sigmoidal Emax model described by the following equation,

$$E = (E_{max} * C^{\gamma})/(EC_{50} + C^{\gamma}),$$

where E represents the response measured as percent prolongation in PR interval, Emax represents the maximum percent prolongation of PR interval, EC_{50} represents the concentration of S-verapamil that causes half of the maximal effect, and gamma represents the Hill coefficient, or slope of the curve. Mean concentration and response data were also fitted to a simple Emax model which is similar to the above equation, except for the absence of gamma. Because a time lag has been observed for response to verapamil in humans following oral and iv dosing (Eichelbaum and Echizen, 1984; Schwartz, et al., 1989; Abernethy, et al., 1993), post-peak verapamil concentration-response curves were also compared. In addition, a PK/PD Link model was used to compare theoretical effect site concentrations to verapamil response. An open two-compartment PK model with first-order absorption and elimination from the central compartment was linked to a sigmoid Emax PD model to predict drug concentrations at a theoretical effect compartment. The pharmacodynamic parameters calculated were Emax, EC_{50}, gamma, and K_{e0}, which represents the lag time between plasma and effect compartment concentrations. The model assumes that R-verapamil does not influence the pharmacokinetics of S-verapamil and does not significantly interfere with the pharmacodynamic response of S-verapamil.

Data is presented as mean ± standard error of the mean. Differences between study arms were tested using the two-way analysis of variance, followed by Bonferroni's post-test for two or more means. Statistical significance was set at $p<0.05$. For TNFα, comparison of the number of plasma samples above the minimum detectable concentrations were analyzed by x^2 test, since fewer than 3 samples in the healthy control group were above minimum detectable concentrations.

Results

Baseline characteristics of study subjects are shown in Table 6-1. There were no significant differences between groups in age, weight, height and body mass index. Baseline systolic blood pressure, diastolic blood pressure, mean arterial pressure and heart rate were also not significantly different between groups. Five patients in the control group reported taking ASA, celecoxib or rofecoxib on an as needed basis for analgesia.

One patient in the healthy control arm was taking propranolol prior to the study. Although non-antiarthritic medications were stopped at least 24 h prior to taking the study medication, propranolol has a steady state half-life of approximately 3-6 h for immediate release and 12 h for controlled release formulations, suggesting that the drug may still have been present in the patient's systemic circulation at the time of our study. Propranolol may potentiate the effects of verapamil on PR interval and blood pressure due to antagonism of beta-adrenergic signaling, however, in this patient, response to the single dose of verapamil was less than for the mean the control group at every measured time point suggesting that propranolol did not have a meaningful additive effect on PR interval.

In the infliximab-treated arm, one patient was taking sotalol prior to the study. At steady state sotalol has a half-life of approximately 12 h, suggesting that the drug was likely to still be present in the patient's systemic circulation at the time of our study. Sotalol is a class III anti-arrhythmic with the QT and PR interval prolongation effects. Sotalol therefore has the potential to interact with verapamil by enhancing the PR interval prolongation and blood pressure lowering effects of verapamil. In this patient, maximal response to the single dose of verapamil was similar to the mean maximal response for this patient's test group, suggesting that sotalol did not influence the findings in this patient group with regards to PR interval or blood pressure.

Rheumatoid arthritis activity was determined by arthritic index and serum pro-inflammatory mediators concentration. Disease activity was well controlled in patients taking infliximab (arthritic index, 2.8 ± 1.3; Table 6-1). In the non-infliximab RA group, disease severity was only available for 3 of 8 patients due to incompletely filled out case report forms. In the 3 patients for whom disease severity was obtained, disease activity was well controlled in 2 patients (arthritic index, 2) and the third had severe disease (arthritic index, 6). Nevertheless, pro-inflammatory mediators concentrations in non-infliximab RA patients suggested that disease was well controlled. Serum nitrite concentrations were not significantly different among the three groups (Figure 6-1). Serum concentrations of TNFα were above the minimum detectable limit in only 1 of 15 healthy subjects, in 0 of 8 non-infliximab patients and in 11 of 12 infliximab patients (x^2, $p<0.001$). Serum C-reactive protein and IL-6 concentrations were significantly greater in RA patients treated with non-infliximab medications compared to control subjects (Figure 6-1), however, they were substantially lower than concentrations encountered in active RA patients reported in the literature (Figure 6-1). CRP and IL-6 concentrations were not significantly different between RA patients taking infliximab and control subjects.

Mean plasma verapamil enantiomer concentration-time profiles are shown in Figure 6-2. S-verapamil concentrations at 12 h were below the limit of detection in all except one subject in each group, hence, plasma concentration-time profile of S-verapamil are only depicted up to 8 h for this enantiomer (Figure 6-2). Plasma verapamil concentrations were not significantly different in RA patients taking infliximab compared with control subjects (Figure 6-2). Mean plasma concentrations as well as the total and unbound AUC of both enantiomers of verapamil were higher – though non-significantly – in non-infliximab RA patients compared with control subjects, due to plasma concentrations for 1 patient that were 2 standard deviations greater than for the mean for that group (Figure 6-2 and Table 6-2). Non-compartmental pharmacokinetic indices and verapamil protein binding are listed in Table 6-2. No significant differences were found in protein binding or pharmacokinetic indices between the three groups.

The time-course of PR interval prolongation following administration of verapamil is shown in Figure 6-3. Mean changes in PR interval prolongation, heart rate, systolic and diastolic blood pressure and mean arterial pressure, expressed as area under the percent change from baseline and maximum percent change from baseline, following administration of verapamil, are shown in Figure

6-4. A large degree of variability was observed for all measured cardiovascular responses to verapamil – therefore, although reduced PR interval prolongation was observed in both groups of RA patients compared with control subjects, the differences were not statistically significant.

The relationship between PR interval and verapamil concentration was analyzed graphically by plotting observed changes in PR interval versus observed plasma concentration (Figure 6-5A) and fitting the data to Emax pharmacodynamic models (Figure 6-6). Variability of estimated Emax and EC_{50} values was large, however, due to the presence of a hysteresis which was more pronounced in the control group than in either RA group (Table 6-3, Figure 6-5A). Comparison of post-peak (1 to 12 h) plasma concentrations vs response resulted in more precise estimates for Emax and EC_{50} in control subjects and the concentration-effect relationship was best described by the sigmoid Emax model (Table 6-3). In contrast, the concentration-effect relationship for the non-infliximab treated RA group was best described by the simple Emax model and was not influenced by absorption phase plasma concentrations, since a hysteresis was not observed for this group (Table 6-3, Figure 6-5). For the infliximab-treated RA group, the concentration-effect data fit best with the sigmoid Emax model when absorption phase plasma concentrations were omitted (Table 6-3). The hysteresis observed for the concentration-effect relationship in control subjects, which indicates a time delay between the plasma concentrations and cardiovascular response, was collapsed when S-verapamil concentrations at the theoretical effect site were plotted versus effect (Figure 6-5B). The relationship between PR interval and effect site concentration are shown in Figure 6-6C and the resulting PD parameters estimated through PK/PD modeling are listed in Table 6-3. Precision of Emax and EC_{50} values were improved using the effect concentration model compared with values estimated using plasma concentrations. A reduced maximal response to the PR interval prolongation effect of verapamil was observed in both groups of RA patients compared with control subjects. EC_{50} and gamma values were comparable between the three groups.

Concentration-time profiles and a PK/PD analysis for individual patients are shown in Appendix 1. The relationship between unbound S-verapamil concentration and PR interval prolongation is shown in Appendix 2.

Table 6-1. Characteristics of Subjects

	Control	RA (Infliximab)	RA (non-Infliximab)
Age (years)	55 ± 2	55 ± 3	61 ± 5
Male/female ratio	1/11	5/7	3/5
Weight (kg)	73.8 ± 5.2	79.3 ± 5.8	75.7 ± 4.6
Height (cm)	161.5 ± 2.4	168.2 ± 3.0	160.9 ± 2.8
Body mass index	28.2 ± 1.6	27.8 ± 1.2	29.5 ± 2.2
Baseline			
SBP (mmHg)	127.9 ± 4.0	131.4 ± 2.8	138.3 ± 6.2
DBP (mmHg)	75.1 ± 2.7	77.3 ± 2.6	76.4 ± 2.7
MAP (mmHg)	95.8 ± 3.3	95.5 ± 2.7	100.1 ± 3.0
Baseline HR	66.2 ± 2.6	61.3 ± 2.8	69.9 ± 4.5
Arthritic Index	0	2.8 ± 1.3	3.3 ± 2.3[a]
Medications (no. of patients)			
Anti-inflammatory	NSAIDs (5)[b]	NSAIDs (7) methotrexate (10)	mtx + leflunomide + ibuprofen (1) mtx + hydroxychloroquine (1) mtx+ prednisone (1) mtx + sulfasalazine + nabumetone (1) hydroxychloroquine (2) diclofenac + prednisone (1)

Data are presented as mean ± s.e.m.

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

[a] Arthritic index available for 3 out of 8 patients; nevertheless, all patients were in remission

[b] Patients reported taking ASA, celecoxib or rofecoxib as needed for analgesia

TABLE 6-2. Non-compartmental pharmacokinetic indices of verapamil enantiomers following single oral 80 mg doses of rac-verapamil.

	Control	RA (Infliximab)	RA (non-Infliximab)
t_{max} (h)			
S	1 (0.5-1.5)	1 (0.5-3)	1 (0.5-4)
R	1 (0.5-1.5)	1 (0.5-1.5)	1 (0.5-3)
C_{max} (ng/mL)			
S	22 ± 6	16 ± 3	25 ± 8
R	93 ± 19	81 ± 14	112 ± 33
$t_{1/2}$ (h)			
S	4.3 ± 0.9	3.9 ± 0.7	4.8 ± 0.6
R	5.0 ± 0.7	5.1 ± 0.5	8.4 ± 2.8
$AUC_{0\text{-}8h}$ (μg.min/mL)			
S	3.2 ± 0.7	3.0 ± 0.6	5.4 ± 2.0
R	17.7 ± 3.6	17.5 ± 4.1	26.9 ± 8.5
$AUC_{0\text{-}\infty}$ (μg.min/mL)			
S	4.9 ± 1.1	4.6 ± 0.9	7.9 ± 2.3
R	22.5 ± 4.8	22.1 ± 5.8	40.6 ± 11.7
CL/F (L/h)			
S	1328 ± 684	774 ± 136	396 ± 50
R	172 ± 36	156 ± 20	113 ± 40
Fraction unbound			
S	0.053 ± 0.004	0.059 ± 0.005	0.053 ± 0.004
R	0.031 ± 0.002	0.034 ± 0.004	0.029 ± 0.003
$AUC_{0\text{-}8h}$ unbound (μg.min/mL)			
S	0.16 ± 0.03	0.17 ± 0.04	0.29 ± 0.10
R	0.51 ± 0.10	0.60 ± 0.16	0.78 ± 0.24
$AUC_{0\text{-}\infty}$ unbound (μg.min/mL)			
S	0.24 ± 0.05	0.27 ± 0.06	0.41 ± 0.11
R	0.65 ± 0.13	0.76 ± 0.22	1.18 ± 0.34

Data are mean ± standard error of the mean, except t_{max} values which are median (range)
No significant difference was found between groups

TABLE 6-3. Pharmacodynamic indices for mean S-verapamil concentrations vs effect relationship. Plasma (A) and post-peak plasma (B) concentrations were fitted to simple and sigmoid Emax pharmacodynamic models. C) Mean S-verapamil concentrations were fitted to a 2 compartment pharmacokinetic model linked to a theoretical effect compartment. Predicted effect compartment concentrations were fitted to a sigmoid Emax pharmacodynamic model.

		Control	RA (Infliximab)	RA (non-Infliximab)
A) Plasma concentration vs effect				
Simple Emax model	Emax (%)	83.8 ± 268.0	23.0 ± 35.3	13.0 ± 6.0
	EC_{50} (ng/mL)	98.8 ± 357.9	27.1 ± 58.4	14.7 ± 13.1
	r^2	0.9018	0.8350	0.8919
	AIC	26.9	18.3	18.6
	SBC	26.8	17.9	18.5
Sigmoid Emax model	Emax (%)	104.7 ± 1600	11.6 ± 19.2	12.4 ± 17.0
	EC_{50} (ng/mL)	84.4 ± 1377	8.8 ± 21.6	13.3 ± 36.6
	gamma	1.2 ± 1.5	1.5 ± 2.2	1.0 ± 1.0
	r^2	0.9029	0.8379	0.8920
	AIC	28.3	20.2	20.6
	SBC	28.1	19.6	20.4
B) Post-peak (1-12h) *plasma* concentration vs effect				
Simple Emax model	Emax (%)	93.0 ± 176.9	24.0 ± 40.5	13.3 ± 6.9
	EC_{50} (ng/mL)	98.9 ± 212.8	27.5 ± 64.9	14.9 ± 14.8
	r^2	0.9822	0.8466	0.8873
	AIC	17.3	15.6	16.4
	SBC	16.9	14.8	16.0
Sigmoid Emax model	Emax (%)	17.3 ± 3.7	10.5 ± 13.1	12.3 ± 18.2
	EC_{50} (ng/mL)	9.6 ± 2.1	7.4 ± 12.3	12.8 ± 38.0
	Gamma	2.6 ± 0.7	1.8 ± 2.7	1.1 ± 1.2
	r^2	0.9918	0.8537	0.8875
	AIC	11.2	17.4	18.4
	SBC	10.6	16.2	17.8
C) Predicted *effect* concentration vs effect - *PK/PD Link Model*				
Sigmoid Emax model	Emax (%)	20.4 ± 8.8	11.2 ± 15.8	12.3 ± 16.3
	EC_{50} (ng/mL)	11.7 ± 5.6	8.4 ± 15.4	12.7 ± 32.6
	gamma	2.0 ± 0.7	1.7 ± 2.6	1.1 ± 1.1
	K_{e0}	7.1 ± 1.5	7.6 ± 14.0	57.4 ± 240.0
	r^2	0.9903	0.8636	0.9056
	AIC	14.5	21.3	21.7
	SBC	14.2	20.5	21.5

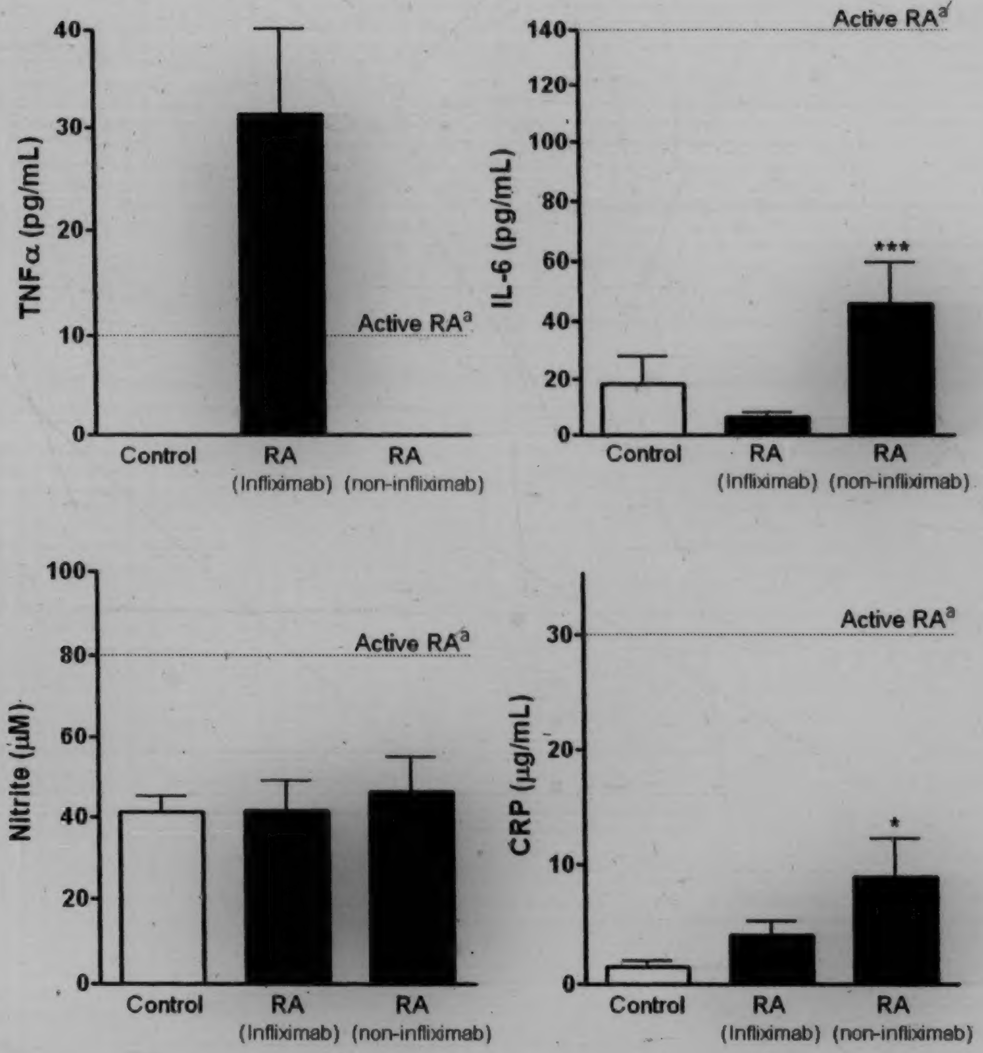


FIGURE 6-1. Mean serum tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), nitrite (NO_2^-), and C-reactive protein (CRP) concentrations at time 0 h in control subjects (n=12), RA patients taking infliximab (n=12) and RA patients taking other anti-rheumatoid therapies (n=8). Error bars represent standard error of the mean. $*p \leq 0.05$, $***p \leq 0.001$. [a]Dotted line shows the relevant physiological concentrations of respective pro-inflammatory mediators encountered in 'Active RA' as reported in references (Charles, et al., 1999; Daneshtalab, et al., 2004; Mayo, et al., 2000).

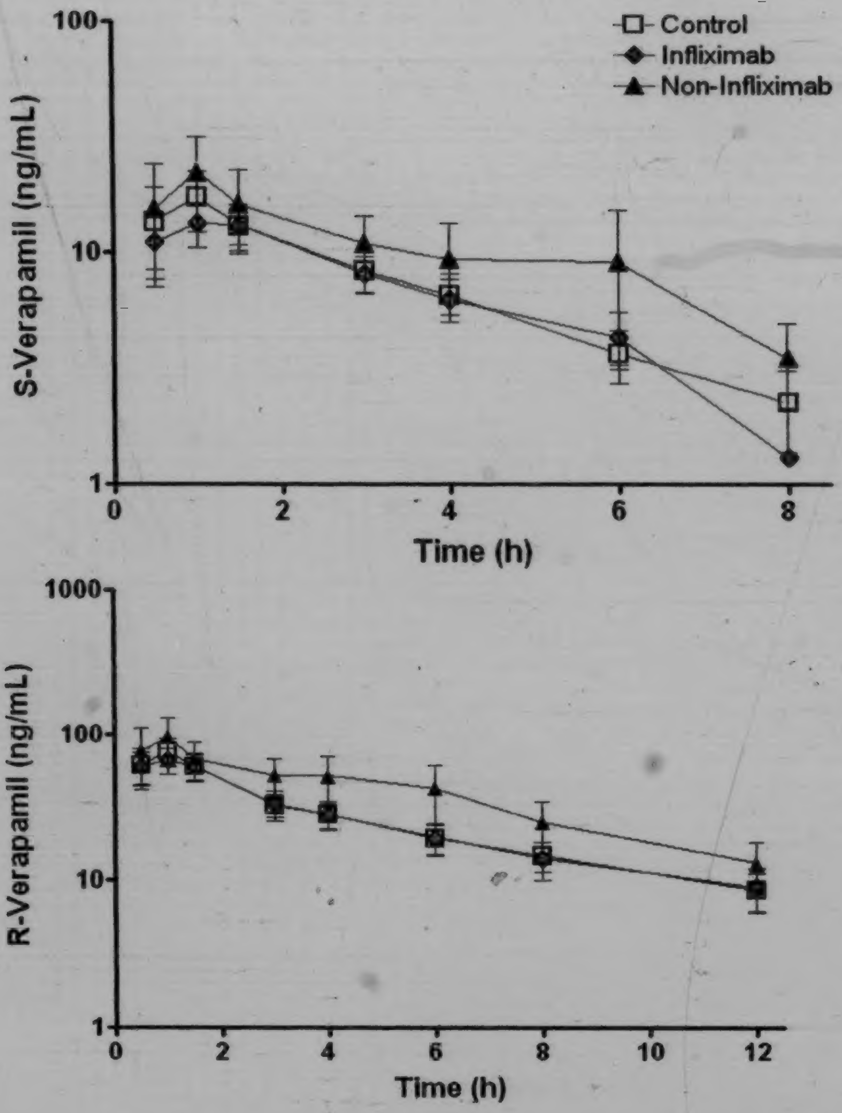


FIGURE 6-2. Mean plasma S- and R-verapamil concentration-time profiles in control subjects (n=12), RA patients taking infliximab (n=12) and RA patients taking other anti-rheumatoid medications (n=8) after a single oral 80 mg dose of verapamil. Error bars represent standard error of the mean. Lines connect experimental data points.

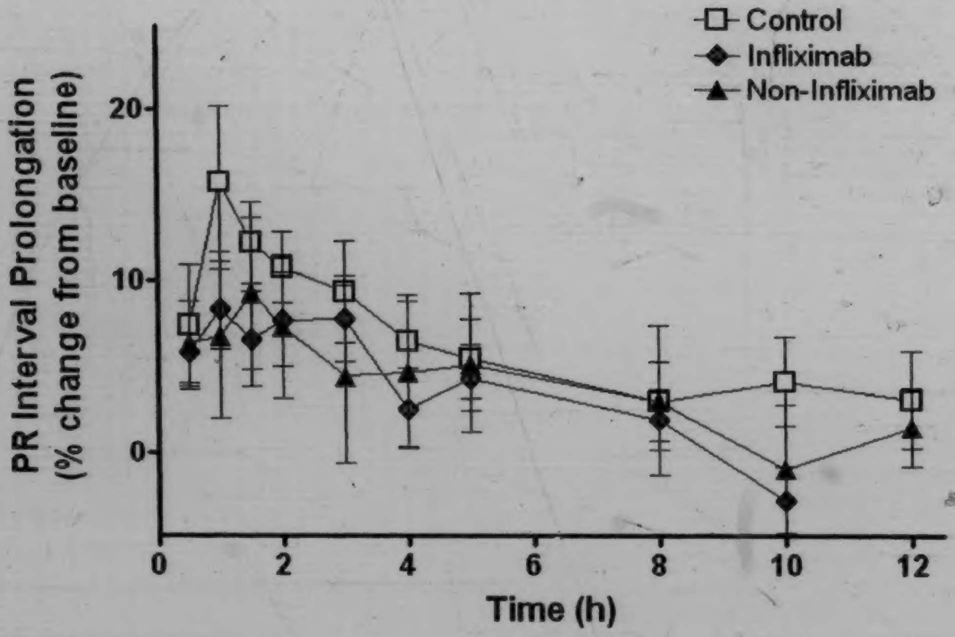


FIGURE 6-3. Time course of mean PR interval prolongation following administration of a single oral 80 mg dose of verapamil in control subjects (n=12), RA patients taking infliximab (n=12) and RA patients taking other anti-rheumatoid medications (n=8). Error bars represent standard error of the mean. Lines connect experimental data points.

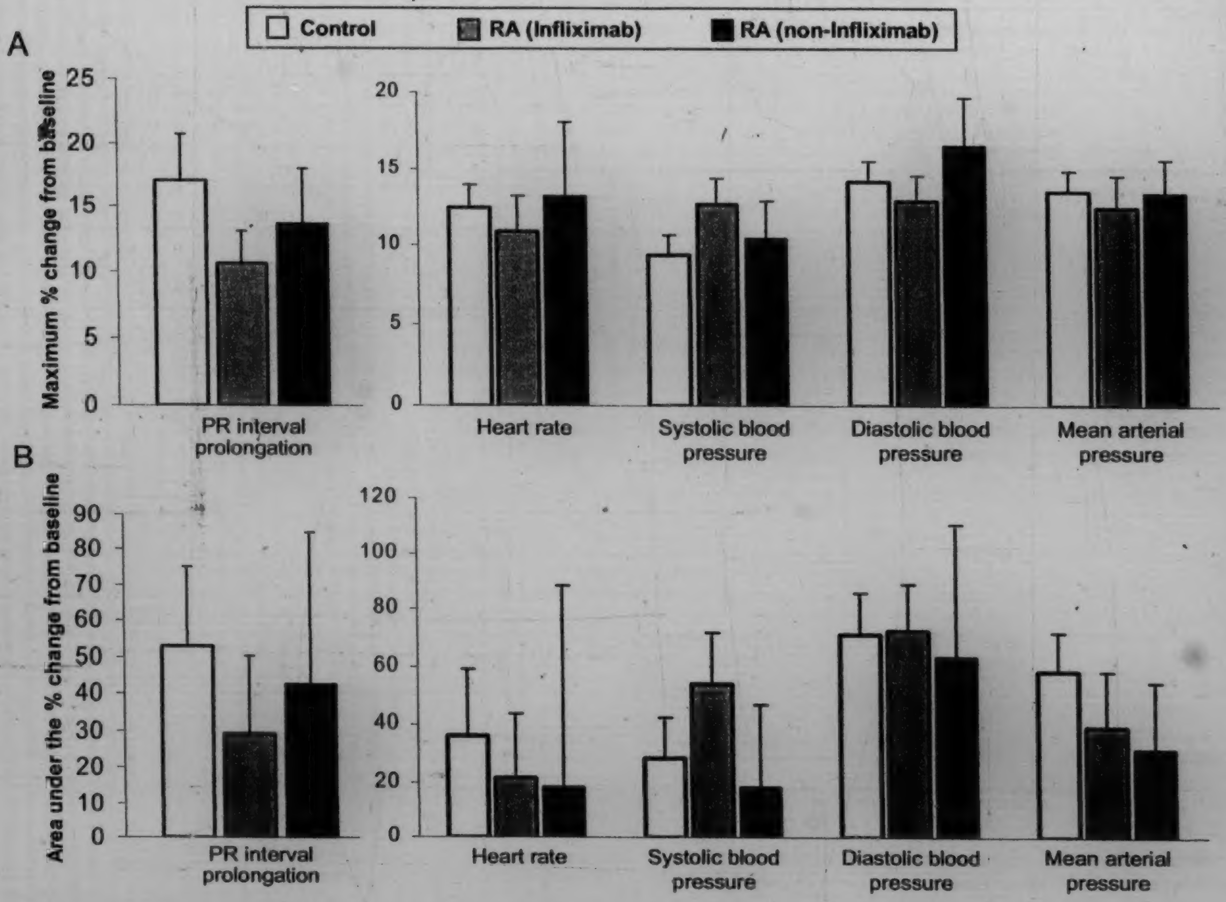


FIGURE 6-4. A) Maximum percent change and B) area under the percent change from baseline, for PR interval prolongation, heart rate, systolic and diastolic blood pressure and mean arterial pressure in control subjects (n=12), RA patients taking infliximab (n=12) and RA patients taking other anti-rheumatoid medications (n=8). Error bars represent standard error of the mean.

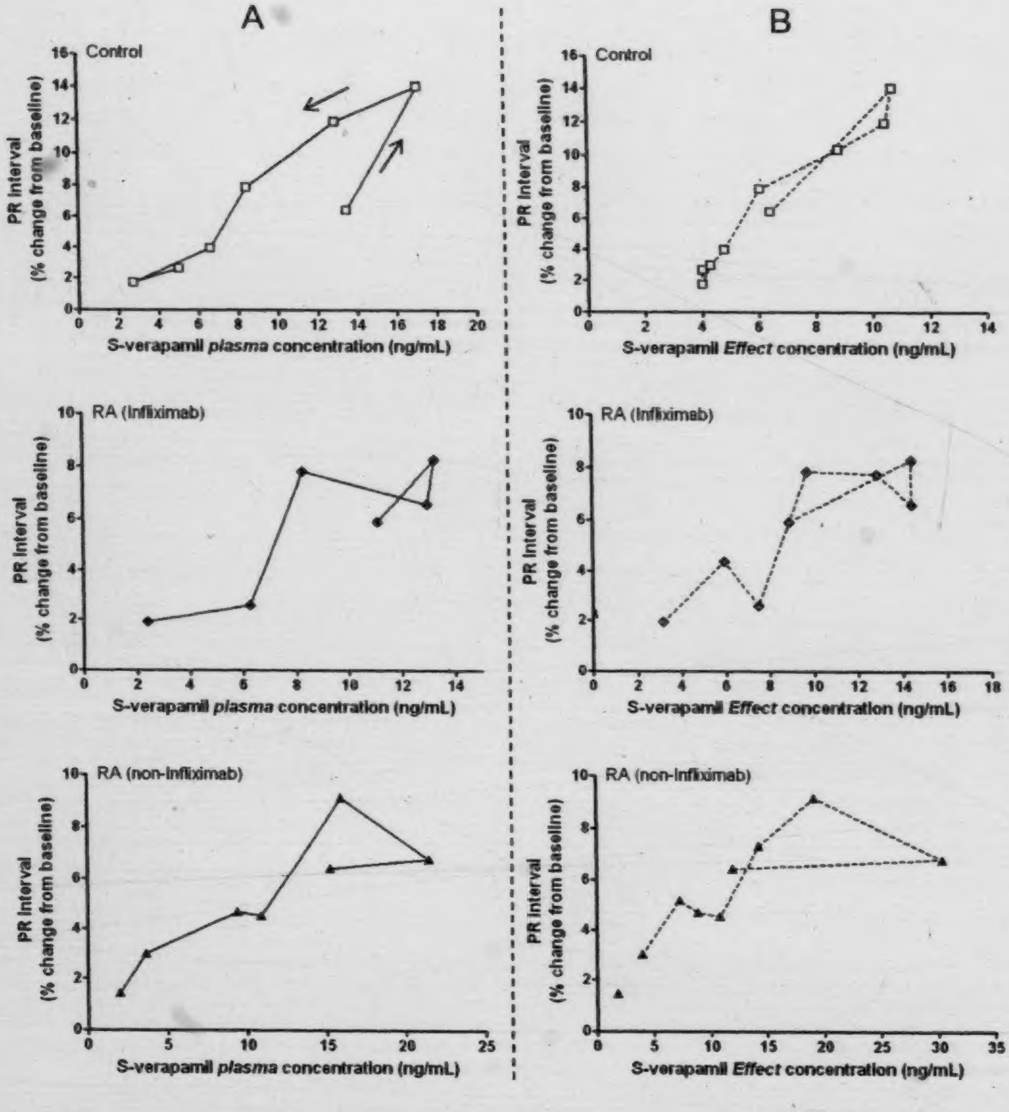


FIGURE 6-5. A) Observed S-verapamil concentrations in plasma vs PR interval. A counter-clockwise hysteresis is observed in the relationship between plasma concentrations and PR interval. B) Predicted S-verapamil concentration in the effect compartment vs PR interval. The hysteresis is collapsed when plasma concentrations were fitted to a two-compartment model with oral input linked to a theoretical effect compartment.

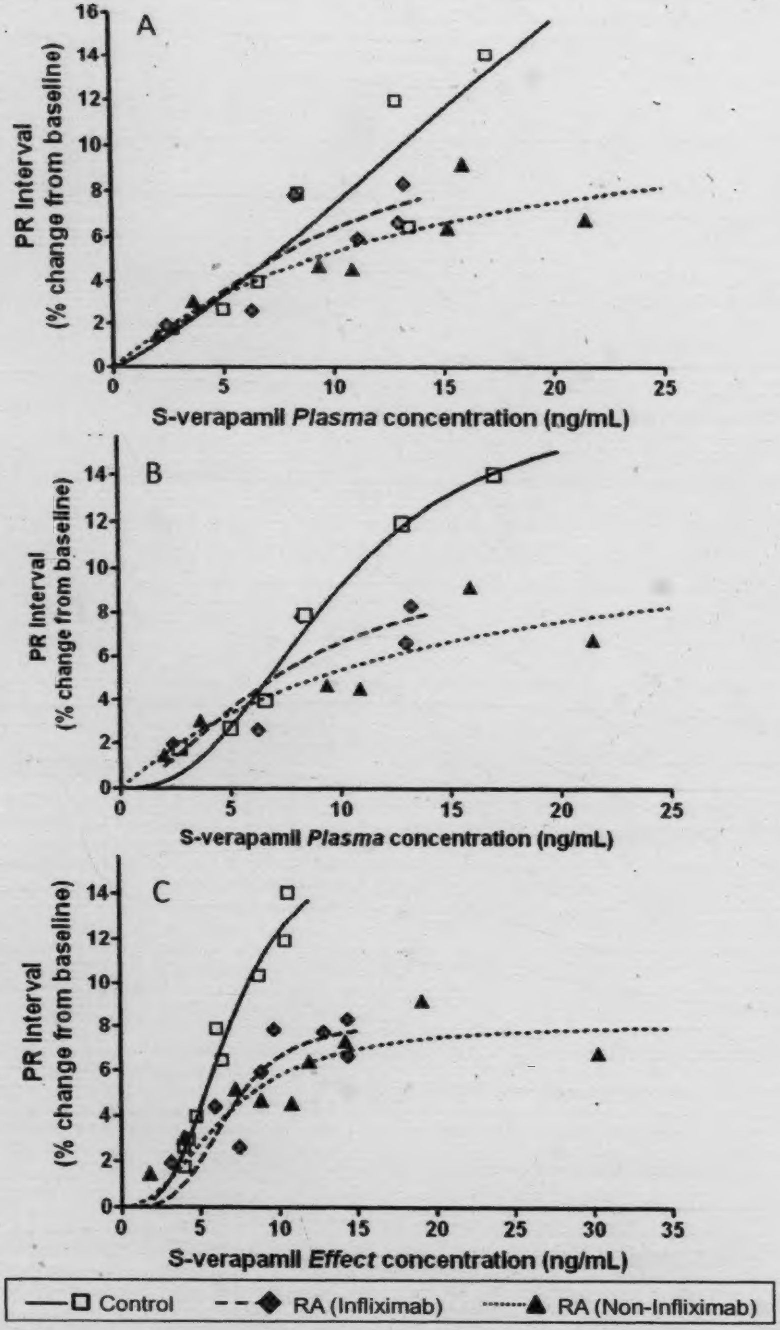


FIGURE 6-6. The relationship between PR interval prolongation and S-verapamil concentrations in plasma (A), post-peak plasma concentrations (B) and at the theoretical effect compartment (C). Regression lines are derived from mean observed plasma concentrations (A and B) and predicted effect concentrations (C) fitted to a sigmoid Emax pharmacodynamic model.

Discussion

The purpose of this study was to determine whether disease remission in RA patients taking either infliximab or other anti-rheumatoid medications would be associated with a reversal of the effects of inflammation on verapamil pharmacokinetics and pharmacodynamics. Under inflammatory conditions, altered pharmacokinetics for drugs with intermediate to high hepatic extraction ratios are well documented (Belpaire, et al., 1989; Kendall, et al., 1979; Laethem, et al., 1994; Piquette-Miller and Jamali, 1993; Schneider, et al., 1976) and are linked to disease severity and increased pro-inflammatory mediators (Mayo, et al., 2000; Piquette-Miller and Jamali, 1995; Sattari, et al., 2003). Active RA, for example, is associated with significant elevations in plasma verapamil concentrations and paradoxical reductions in pharmacologic response (Mayo, et al., 2000).

In the present study, remission from active disease appeared to be associated with normal verapamil pharmacokinetics, due perhaps, to reduced levels of inflammation. As expected, we found no significant differences in plasma concentrations and pharmacokinetic indices of either enantiomer of verapamil in either group of RA patients compared with healthy control subjects (Figure 6-2, Table 6-2). This may have been attributable to reduced serum nitrite concentrations in both groups of RA patients towards those of control subjects (Figure 6-1). Under inflammatory conditions, serum nitrite is associated with disease severity and increases in verapamil AUC (Mayo, et al., 2000; Sattari, et al., 2003). CRP and IL-6 levels were also not significantly different among RA patients treated with infliximab compared with controls (Figure 6-1). Among RA patients taking non-infliximab anti-rheumatoid medications, CRP and IL-6 concentrations were significantly higher than in control subjects; however, these were still substantially lower than concentrations encountered in active RA patients reported in previous studies and are reflective of good disease control (Figure 6-1) (Charles, et al., 1999; Daneshtalab, et al., 2004; Mayo, et al., 2000; McEntegart, et al., 2001). By comparison, patients with active RA have elevated levels of pro-inflammatory cytokines including IL-1β, IL-6, TNFα, and NO (Charles, et al., 1999; Ueki, et al., 1996). Interestingly, serum levels of TNFα were detectable in only 1 of 12 control subjects, in none of the 8 non-infliximab treated patients but in 11 of 12 infliximab treated patients (x^2, $p<0.001$). This finding may be explained by the inherent nature of TNFα and the method of detection. Measurement of TNFα in serum poses a challenge, as endogenous TNFα is present in very low concentrations, has a short half-life, and is affected by several factors including sample handling and the detection method used. Systemic levels of TNFα vary greatly and mean serum concentrations range from 10 to 30 pg/mL in RA patients as measured by immunoassay (Charles, et al., 1999; Manicourt, et al., 1993). In healthy subjects, serum TNFα is often undetectable (Arvidson, et al., 1994) and can range from <5 to ~20 pg/mL (Candon, et al., 2005; Mangge, et al., 1995). The TNFα assay used in our study has a limit of detection in serum of 2 pg/mL and the manufacturer reported that levels of TNFα were below detectable limits in serum or plasma in 50 of 58 samples tested from healthy individuals. TNFα in the present study was measured by an ELISA that used a polyclonal antibody for the capture antibody and a monoclonal antibody for the detection antibody. This type of immunoassay detects both trimeric and monomeric forms of TNFα as well as TNFα which is free and bound to soluble TNF receptors and does not distinguish between biologically active and inactive TNFα (Corti, et al., 1992; De Groote, et al., 1993; Engelberts, et al., 1991; MacEwan, 2002). High circulating levels of TNFα have been reported following anti-TNFα therapy for up to 4 weeks in RA patients (Charles, et al., 1999) and up to 8 weeks in pediatric Crohn's patients (Candon, et al., 2005); however, the TNFα detected in both studies was determined to be biologically inactive. This phenomenon is not unusual, as high levels of circulating IL-6 have also been seen following induction with anti-IL-6 antibodies (Lu, et al., 1992). Our data clearly demonstrates that high levels of TNFα are also seen during infliximab therapy in RA patients. Since serum levels of nitrite, IL-6 and CRP in both of our RA patient groups were similar to control levels and therefore reflective of good disease control, we believe the TNFα observed in our infliximab treated patients reflects the inactive infliximab-bound protein.

Reduced drug clearance in inflammation may be caused by increases in plasma protein binding and/or suppression of hepatic drug metabolism. Under inflammatory conditions, verapamil protein binding is increased, secondary to elevations of the plasma protein AAG (Belpaire, et al., 1982; McGowan, et al., 1983), resulting in significantly reduced unbound fraction of verapamil (Laethem, et al., 1994; Ling and Jamali, 2005; Mayo, et al., 2000). In the present study protein binding of verapamil was not significantly different in RA patients in remission compared with healthy controls (Table 6-2). Remission from active RA, therefore, may have resulted in a reduction in AAG, which, like CRP is an acute phase protein that increases with disease severity (Nakamura, et al., 1993). Cytochrome P450 is also known to be inhibited under inflammatory conditions (Morgan, 1997), due perhaps, to pro-inflammatory cytokines (Abdel-Razzak, et al., 1993) and NO (Khatsenko, et al., 1993). Infliximab therapy has been shown to reduce levels of pro-inflammatory cytokines and NO (Charles, et al., 1999; Perkins, et al., 1998; Schuerwegh, et al., 2003; Vuolteenaho, et al., 2002). In chapter 5, we demonstrated that suppression of total cytochrome P450, CYP3A and CYP1A protein content by inflammation was reversed to a limited extent by infliximab treatment in a rat model of early adjuvant arthritis (Figure 5-7). Our present findings of apparently normal plasma verapamil levels suggests hepatic metabolism was not inhibited in RA patients in remission as has been suggested in patients with active RA (Mayo, et al., 2000). Notably, in our current study, both groups of patients in remission from RA showed plasma concentrations of verapamil similar to that of healthy controls, indicating that disease remission, and the associated reduction of pro-inflammatory mediators, rather than infliximab treatment specifically, resulted in a reversal of inflammation-induced decreases in verapamil clearance.

Interestingly, our data suggests an altered drug concentration-effect relationship in RA patients following verapamil administration despite treatment with infliximab or other anti-rheumatoid medications. Under inflammatory conditions such as RA, the dromotropic effect of verapamil is reduced; the potency to prolong PR interval is diminished by 50 % (Mayo, et al., 2000). Other conditions such as old age and obesity that are associated with elevated pro-inflammatory mediators also result in reduced response to verapamil (Abernethy and Schwartz, 1988; Abernethy, et al., 1986; Abernethy, et al., 1993). In addition, reduced PR and QT interval prolongation has also been reported for the beta-adrenergic antagonist propranolol and beta and potassium blocker sotalol in various rat models of inflammation (Guirguis and Jamali, 2003; Kulmatycki, et al., 2001). In the present study, maximum percent change in PR interval as well as area under the percent change from baseline for PR interval was reduced by ~40% in infliximab treated RA patients, although the differences were not statistically significant (Figure 6-4). The time-course of PR interval prolongation following verapamil administration also showed a non-significant reduction in response to verapamil in both groups of RA patients (Figure 6-3). Furthermore, the magnitude of response was consistent with those previously observed in healthy and RA patients (Mayo, et al., 2000). Mayo et al, documented a significant reduction in response to verapamil in RA patients despite a small sample size of only 16 patients, demonstrating a strong inhibitory effect of inflammation in this patient population. The lack of significance in our study is perhaps explained by the correlation between disease severity and the magnitude of effect of inflammation (Mayo, et al., 2000). In our study RA patients were in remission; as a result, we observed greater variability in disease severity and thereby greater variability in the associated effects of inflammation – hence reduced power to detect a statistically significant effect.

The potency of verapamil to prolong PR interval was assessed by examining the concentration-effect relationship. Plots of mean S-verapamil concentration versus percent change in PR interval showed increased prolongation of PR interval with increasing plasma drug concentrations (Figure 6-5A). Large variability of Emax and EC_{50} values were estimated (Table 6-3), particularly in healthy controls, however, due to a counter-clockwise hysteresis observed between plasma S-verapamil concentrations and PR interval prolongation, suggesting an indirect relationship due, perhaps, to a time-delay between plasma concentration and pharmacologic effect (Figure 6-5A). The hysteresis was collapsed after fitting the data to a PK/PD link model to predict S-verapamil concentrations at a theoretical effect compartment (Figure 6-5B). Estimated K_{e0} values (Table 6-3) indicate a time lag of approximately 10, 9 and 1 min between plasma and effect site concentrations in control, infliximab-treated and non-infliximab treated RA groups, respectively. Using a sigmoidal Emax pharmacodynamic model, effect site concentrations showed a direct relationship with PR interval (Figure 6-6C). Precision of the pharmacodynamic model was improved greatly using predicted effect concentrations compared with observed plasma concentrations (Table 6-3). Maximal response (Emax) to verapamil was reduced from 20.4 ± 8.8 for healthy controls to 11.2 ± 15.8 and 12.3 ± 16.3 in RA patients treated with infliximab and other anti-rheumatoid medications, respectively (Table 6-3). Comparison of post-peak verapamil plasma concentration vs effect likewise improved the precision of pharmacodynamic models describing the concentration-effect relationship. It is of interest to note that our data showed similar Emax and EC_{50} estimates using the sigmoid Emax model when comparing both post-peak concentration vs effect as well as effect site concentration vs effect. It has been previously shown that predicted effect site concentrations of verapamil are more predictive of the steady state concentration-effect relationship than are post-peak plasma concentration (Schwartz, et al., 1989). Our findings suggest disease remission in RA patients is not associated with a complete reversal of pharmacodynamics, despite reduced systemic levels of pro-inflammatory mediators and a reversal of the effects of inflammation on pharmacokinetics.

Three explanations are possible for our observations: 1) negative effects of the infliximab/TNFα complex on the heart, 2) inflammation-induced damage to the heart is irreversible or only very slowly reversible, or 3) some pathology other than increased pro-inflammatory mediators is responsible for reduced sensitivity of calcium channel receptors. First, although we believe the high levels of TNFα measured in our study reflect the bound, inactive form of TNF, the infliximab/TNF complex may cause deleterious effects on the heart. The activation of complement and subsequent cell lyses caused by the binding of infliximab to surface TNF expressed on myocytes has in fact been proposed as an explanation for the negative impact of infliximab therapy in patients with heart failure (Sarzi-Puttini, et al., 2005). Likewise, etanercept, a recombinant soluble TNFα receptor fusion protein, also binds TNF, but due to its fast dissociation rate, effectively prolongs the half-life of TNF and increases its concentration in serum, where it may result in the worsening of heart failure (Culy and Keating, 2002). It is possible that the presence of infliximab/TNF complexes and/or dissociation of infliximab/TNF complexes exerted inhibitory effects on cardiac response to verapamil in RA patients in our study. Nevertheless, neither scenario fully explains our findings, as reduced response to verapamil was also seen in RA patients not taking infliximab and in whom serum TNF was undetectable.

Second, deleterious effects of inflammation on cardiac myocytes or calcium channel functioning may be irreversible or very slowly reversible. Down regulation or desensitization of cardiac receptors can occur in a number of ways. Pro-inflammatory cytokines may affect cardiac function directly or indirectly through effects on β-adrenoreceptors or through stimulation of NO. TNFα exerts negative inotropic effects on cardiomyocytes by NO-dependent (Finkel, et al., 1992) as well as NO-independent (Muller-Werdan, et al., 1997) pathways. Low concentrations of TNFα have been shown experimentally to activate the sphingomyelin pathway which in turn modifies calcium currents by inhibition of L-type calcium channels (Oral, et al., 1997; Sugishita, et al., 1999). Our lab has demonstrated the binding capacity of L-type calcium channels is significantly reduced in the presence of elevated serum nitrite and TNFα in the inflamed rat heart (Sattari, et al., 2003). In addition, the L-type calcium channel, to which verapamil binds, is controlled in part by β-adrenergic receptors (Liu, et al., 1999; Zhou, et al., 1997). Desensitization of β-adrenoreceptors by inflammation has been reported in RA (Baerwald, et al., 1992), heart failure (Bavendiek, et al., 1996) and asthma (Moore, et al., 2001). Reduced β-receptor density has been seen in peripheral blood mononuclear cells of RA patients where the decrease was related to disease activity (Baerwald, et al., 1992), and in cardiac myocyte and airway smooth muscle cell due to presence of TNFα and IL-1 (Gulick, et al., 1989; Moore, et al., 2001). It is perhaps through down-regulation of β-receptors that the response to drugs such as the beta antagonist propranolol and calcium channel blocker verapamil is reduced. Experimentally, treatment with anti-TNFα antibody has been shown to reverse the suppressive effects of inflammation on the inotropic response in cardiomyocytes (Boekstegers, et al., 1996) and myocardial pressure and vascular adrenergic response to endotoxic shock in the perfused rat hearts (Boillot, et al., 1997). It is unknown to what extent inflammatory effects on cardiac receptor function are reversible or how long patients must be free of inflammation for reversal of effects to be manifest. The RA patients in the present study reported having arthritis for an average of 15 years before participating in this study. Negative consequences of their disease on cardiovascular function therefore may require prolonged treatment to reverse.

Third, disease processes in RA involving pathologies other than pro-inflammatory mediators may have resulted in altered receptor function in parallel to inflammatory changes rather than be driven by inflammatory changes. For example, down regulation of the adrenergic system has been explained as an adaptive response to cardiac injury following myocardial infarction and in heart failure, where chronic or over stimulation of sympathetic nervous system leads to progression of the failing heart (Sawyer and Colucci, 1998). Down-regulation of NO-sensitive cardiac receptors in RA may be an adaptive response to chronic systemic inflammation where cytokine-stimulated overproduction of NO leads to direct cardiodepressant effects.

In summary, remission from active disease appears to restore plasma protein levels and hepatic drug metabolism activity in patients with RA, resulting in relatively normal verapamil pharmacokinetics. This may be explained by low levels of pro-inflammatory mediators concentrations, which are associated with altered pharmacokinetics at higher concentrations such as those observed under inflammatory conditions. There was no corresponding reversal of the effects of inflammation on PR interval prolongation effects of verapamil. This may reflect a more pathologic dysfunction of cardiac receptors caused by chronic RA prior to remission, rather than an on/off effect with regards to the presence or absence of inflammatory mediators. There were no clinically significant differences between RA patients taking infliximab compared with patients taking other anti-rheumatoid medications. This perhaps reflects the similarity in current RA therapies at reducing T-cell driven inflammation as well as the failure to resolve the underlying autoimmune pathology.

CHAPTER 7

Summary and Conclusions

As the prevalence of cardiovascular and rheumatic diseases increases with an aging population, the potential for interactions – both drug-drug and drug-disease – also increases. While the characterization of drug action and disposition has traditionally been carried out in the healthy adult population or in patients with a single disease, it is becoming increasingly clear that the presence of multiple diseases can profoundly influence pharmacokinetics and pharmacodynamics. Disease-drug interactions involving inflammatory conditions such as cardiovascular and arthritic disease are especially relevant in this context and pose a difficult challenge as well as a unique condition for the concurrent treatment of both conditions. Besides the heightened inflammatory state that predisposes to the development of atherosclerotic conditions, RA potentially increases cardiovascular risk through reducing response to antihypertensive therapy. Hypertension is an established risk factor for myocardial infarction and stroke, and is associated with an elevated pro-inflammatory cytokine profile. Patients with RA commonly develop hypertension due to inflammation, as a result of adverse effects common to anti-inflammatory treatment with NSAIDs and also perhaps due to reduced response to cardiovascular drugs such as β-blockers and calcium channel antagonists. When altered drug clearance in patients with diseases such as diabetes, hypertension, and rheumatoid arthritis, is considered along with their unexpected results, then the therapeutic outcome of multiple drug therapy is no longer straight-forward.

Since a similar pathology underlies the development of both cardiovascular and arthritic diseases, then perhaps, similar strategies may provide therapeutic benefit or protection against the progression and mortality of disease. Much recent research has focused on elucidating this perspective. Cardiovascular drugs such as the ACE inhibitors, ARBs and statins have proven unexpectedly beneficial for reduction of cardiovascular morbidity and mortality due to anti-inflammatory effects independent of their anti-hypertensive or cholesterol lowering properties. Evidence is also emerging to suggest a cardiovascular risk reduction in arthritic patients being treated with the anti-rheumatic drugs methotrexate and infliximab. The reasons for improved cardiovascular disease profile with anti-inflammatory therapy are undoubtedly multi-faceted, but among them may be the effects of decreased inflammation on factors affecting drug disposition and consequently, response to drug therapy.

The goal of our work was to examine whether anti-inflammatory treatment would be associated with reductions in systemic inflammation and whether that would result in a reversal of the inhibitory effects of inflammation on pharmacokinetics and pharmacodynamics in rats and humans.

For rat studies, we first evaluated the impact of cannulation surgery on the stress response in healthy rats. A common procedure in animal experimentation is catheterization of blood vessels for injection of drugs or sampling of biological fluid while minimizing the stress and handling of the animal. The surgical procedure and the presence of the catheter itself, however, may cause stress and influence various physiological parameters that can influence subsequent experiments. We measured the corticosterone response to acute restraint stress as a measure of animal recovery from the effects of surgery and cannulation. We found baseline corticosterone concentrations to be similar to pre-surgery levels one day following cannulation surgery and a robust corticosterone response to acute restraint stress could be induced at that time, suggesting that HPA-axis recovery was evident as early as one day after surgery. This experiment demonstrated that the stress of surgery was short-lived and did not impact subsequent stress response to a different acute stressor such as restraint stress, which is relevant to animal handling and experimentation. We suggest that since the stress of surgery was minimal 24 h after surgery, then other factors related to stress which may influence pharmacokinetics or inflammatory disease would also be minimal. Since all test animals in subsequent experiments would be subjected to the same surgical procedures, any residual effects of surgery would be reasonably controlled for. Nevertheless, it would be of interest to examine further, the impact of other factors such as plasma protein levels, CYP enzyme content and pro-inflammatory mediators levels following surgery or restraint stress, on pharmacokinetics and pharmacodynamics.

The inhibition of verapamil clearance is well documented in acute and chronic models of inflammation in the rat. In order to assess the ability of infliximab to reverse the effects of inflammation, however, we required a model of disease of long duration, i.e., one that does not spontaneously resolve, therefore, precluding the use of acute inflammatory disease models. In an effort to reduce the pain and distress of the well-established adjuvant arthritis model of chronic inflammation, we first explored the use of the early, pre-arthritic phase of disease for pharmacokinetic studies. We hypothesized that the pre-arthritic disease model would exhibit the effects of inflammation on pharmacokinetics while sparing the animals from the pain and suffering of the full disease. We confirmed that the rise in pro-inflammatory mediators nitrite, CRP, and TNFα occurred within days of adjuvant injection and coincided with significant decreases in verapamil clearance. Furthermore, elevated verapamil concentrations were significantly correlated with inflammatory mediators as well as with reductions in hepatic CYP enzymes. Significantly reduced binding of verapamil to plasma proteins was also observed. Pre-AA therefore represents a suitable model of inflammatory arthritis in rats for the study of pharmacokinetics, as this model exhibits the inflammation-induced alterations in protein binding, drug metabolism and drug clearance observed in both acute and chronic disease. While the chronic effects of AA are well documented, we now show that the early pre-arthritic phase also influences drug clearance due, perhaps, to the combination of increased protein binding and reduced cytochrome P450 enzymes secondary to increased pro-inflammatory mediators. The new model provides the opportunity to conduct studies under the systemic condition of arthritis in the absence of pain and suffering associated with the fully developed adjuvant arthritis.

Our next objective was to determine whether single doses of infliximab injection would decrease systemic pro-inflammatory mediators and reverse effects of inflammation on verapamil metabolism and pharmacokinetics in pre-AA rats. We found hepatic CYP enzymes to be significantly increased 8 days following infliximab injection, although levels were still lower than in healthy controls. Recovery of CYP enzymes with infliximab treatment therefore appears to occur slowly, requiring at least a week of exposure for the effects to be apparent. It is possible, however, that CYP proteins levels did not *recover*, i.e., increase in expression by infliximab after having been suppressed by pre-AA – rather, the infliximab treatment prevented the further suppression of CYP enzymes observed in untreated pre-AA rats. This scenario is unlikely however, as CYP proteins expression in untreated pre-AA rats at day 14 was suppressed to a similar extent to levels seen already at day 6 of pre-AA. CYP protein levels in infliximab-treated rats therefore likely reflected an increase or recovery of hepatic CYP proteins. We expected infliximab treatment to decrease inflammation in pre-AA rats, however, our data is not clear in this regards. Mean pro-inflammatory mediators nitrite and CRP were not significantly reduced by infliximab; however, CYP proteins expression was significantly correlated to serum nitrite concentrations. This finding supports the theory that NO has a prominent role in CYP inhibition, although it is unknown whether the effects of infliximab on CYP protein expression observed in our study was caused by reductions in inflammation. As expected, binding of verapamil to plasma proteins was increased in pre-AA – an effect not reversed by infliximab treatment. Consequently, despite the partial recovery of CYP enzymes, verapamil clearance was unaffected by infliximab. To our knowledge this is the first work to examine the effects of infliximab in an established model of arthritis in the rat. While infliximab has been shown to reduce inflammation in other models of inflammation, we found the influence of infliximab to be limited to CYP protein expression – markers of systemic inflammation and pharmacokinetics were unaltered. Several factors may influence the ability of infliximab to reverse inflammatory changes in the rat; the severity of the pre-AA model of inflammation, the specificity of infliximab for rat TNFα, and the dose and duration of exposure to infliximab. With regards to the latter, single doses of 10 mg/kg were chosen based on preliminary dose-response data. Perhaps with larger or multiple doses of infliximab and for increased duration of treatment, the ability of the drug to reverse inflammatory changes would be more apparent.

It is known that patients with active RA exhibit reduced pharmacologic response to verapamil in spite of increased plasma drug concentrations. The presence of elevated pro-inflammatory mediators in RA is associated with increased drug concentrations and perhaps also causes the down-regulation of response to verapamil. It is unknown whether reversing the pro-inflammatory state in patients with RA would result in a reversal of the adverse effects of inflammation on verapamil pharmacokinetics and pharmacodynamics. We therefore endeavored to examine whether RA patients who were effectively managed with pharmacotherapy, and therefore in clinical remission, would exhibit reduced levels of systemic inflammation and consequently, normal verapamil pharmacokinetics and pharmacodynamics.

Two groups of patients with controlled RA were enrolled: patients treated with infliximab for more than 24 months and patients treated with non-biologic anti-rheumatic medications; as well as a third group of healthy volunteers. Both groups of RA patients exhibited low levels of the pro-inflammatory mediators, nitrite and CRP. This was expected as serum nitrite is positively correlated with arthritic disease severity and the acute phase protein, CRP, is recognized as a sensitive marker of systemic inflammation. In addition, plasma protein binding of verapamil in both groups of RA patients was also similar to that of healthy controls, suggesting that disease remission also results in a reduction of the acute phase protein, AAG. On the other hand, serum concentrations of TNFα were significantly elevated in infliximab treated patients compared with patients not taking infliximab and with healthy subjects. This observation, however, may be explained by the method of detection of TNFα. Commercially available ELISA kits measure monomeric and trimeric forms of TNFα as well as the bound and unbound protein; hence, elevated levels of TNFα measured by ELISA in patients taking infliximab may be due to the bound infliximab/TNFα complex and therefore not biologically active. Elevated cytokine levels during anti-cytokine therapy are not uncommon, and have been documented following anti-IL-6 and anti-TNFα therapy. Reduced levels of inflammation and normal extent of protein binding led to a reversal of the effects of inflammation on verapamil clearance and hence, plasma concentrations of the drug in our patients.

The above finding was in contrast to our findings in a rat model of arthritis, where infliximab treatment did not influence verapamil pharmacokinetics. This discrepancy may be attributed to differences in infliximab dosing or perhaps infliximab specificity for human rather than rat TNF. Regarding the former, single doses of infliximab were administered to rats, whereas RA patients received infusions of the drug every 8 weeks. In addition, only patients in remission, and thus, responders, to their respective anti-rheumatoid therapies were enrolled into the study. In contrast, all rats injected with infliximab were included in the analysis, since the use of pre-AA meant that only asymptomatic animals were included in the analysis. As such there was no clinical measure to determine whether pre-AA rats injected with infliximab showed a response. Serum concentrations of inflammatory mediators were measured, however, to determine the level of systemic inflammation in pre-AA rats. We thus determined that infliximab injection did not significantly reduce inflammation. Furthermore, rats exhibited significant increases in plasma protein binding despite infliximab, suggesting that acute phase proteins remained elevated, whereas protein binding in RA patients was at the level of healthy controls. Nevertheless, infliximab treatment in the rat did reverse, to some extent, the effects of pre-AA with regards to hepatic CYP proteins expression and the increases in protein were significantly correlated with serum nitrite levels. In humans, as in rats, inflammation is expected to have a more pronounced influence on pharmacokinetics of highly extracted drugs after oral administration; even small changes in hepatic enzyme function would produce significant changes in plasma concentrations of drug. The apparently normal plasma concentrations of verapamil in RA patients therefore suggest that hepatic enzyme function was relatively unchanged. This was apparent in patients treated with infliximab as well as in patients treated with non-biologic anti-rheumatoid medication, suggesting that the normalization of verapamil clearance was due to reduced levels of inflammation rather than to infliximab treatment.

In active RA patients, an altered PK/PD relationship exists – reduced drug response despite elevated drug concentration. In our study of patients with controlled RA, a non-significant trend towards reduced PR interval response to verapamil was observed. PK/PD modeling, however, suggests that the potency of verapamil to prolong PR interval was reduced in both groups of patients compared with healthy volunteers. The potentially reduced response occurred in the absence of changes in verapamil pharmacokinetics which supports previous suggestions that inflammation alters drug response via receptor down-regulation, rather than through pharmacokinetic changes such as protein binding or plasma drug levels. That disease remission did not appear to reverse this effect suggests that damage caused by chronic inflammation is not easily repaired and that patients' inflammatory status is an important component of their cardiovascular risk profile.

It is possible that remission induced by infliximab and/or other anti-rheumatoid medications may exert subtle effects on drug response that was not detectable in our study due to the greater variability in our patient groups and to the lack of an active RA arm. It is acknowledged that the magnitude of inflammation's effect on PK/PD is dependent on disease severity. It may be argued that the reduced response to verapamil in our study was smaller in magnitude than in the active RA population, as a result of disease remission. The reduced potency of verapamil to prolong PR interval in active RA patients occurred in the presence of significantly elevated verapamil concentrations. In our study, a similar reduction in verapamil response was observed in the presence of normal drug concentration, thus, the reduction in response relative to drug concentration was less. Whether this interpretation is accurate and if so, whether it was the result of lower inflammation, is not certain. It is interesting to note that infliximab has been shown to reverse the effects of inflammation on sotalol pharmacodynamics in an acute model of inflammation in the rat. The effects of infliximab on verapamil pharmacodynamics have not been studied in the rat. As our data regarding infliximab's effects on verapamil pharmacokinetics in pre-AA rats demonstrates, however, a suitable method of reversing the effects of AA-induced inflammation after its onset has yet to be established.

In summary, this body of work further explores the interactions between inflammatory disease and drug response and disposition. We confirm the appropriateness of the common cannulation procedure in rats for pharmacokinetic studies as well as establish the usefulness of pre-AA as a model of inflammation for pharmacokinetic studies. Elevated pro-inflammatory mediators are associated with altered verapamil pharmacokinetics in rats and humans. Serum nitrite in particular appears strongly correlated with increased drug exposure and decreased hepatic CYP expression. Our findings support the theory that altered drug disposition in inflammation involves the excess production of nitric oxide. Treatment of pre-AA rats with infliximab did not reduce inflammation or inflammation-induced changes in verapamil pharmacokinetics. Nevertheless, infliximab did partially, though significantly, reverse inflammation-mediated suppression of hepatic CYP expression; an effect that was significantly correlated with serum nitrite concentrations. Disease remission in RA, on the other hand, was associated with reduced systemic inflammation and normal pharmacokinetics in patients treated with infliximab and other anti-rheumatoid medications. Inflammation-induced pharmacodynamic changes, however, potentially persist in remission patients, suggesting that altered drug response occurs independent of the pro-inflammatory mediators studied.

CHAPTER 8

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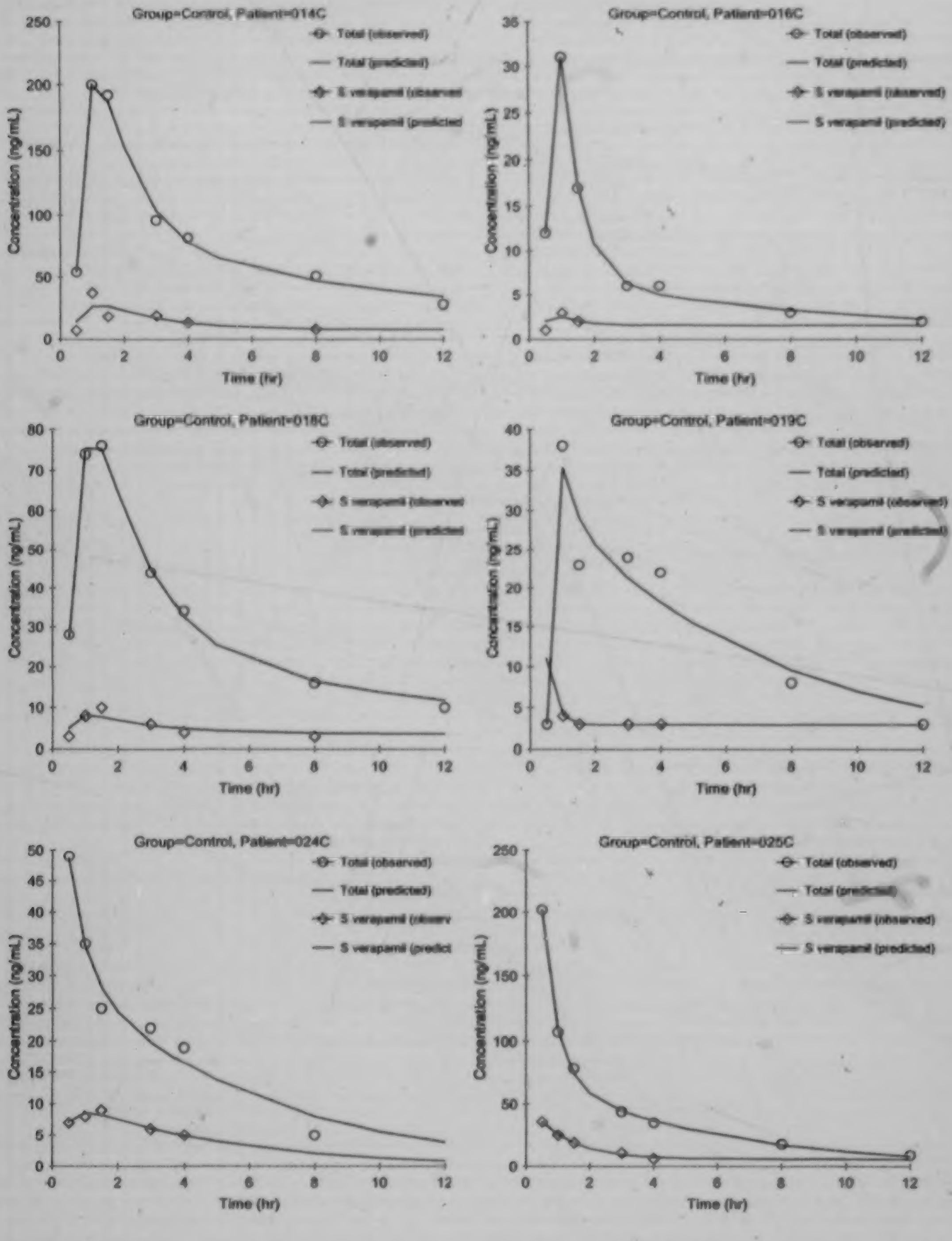
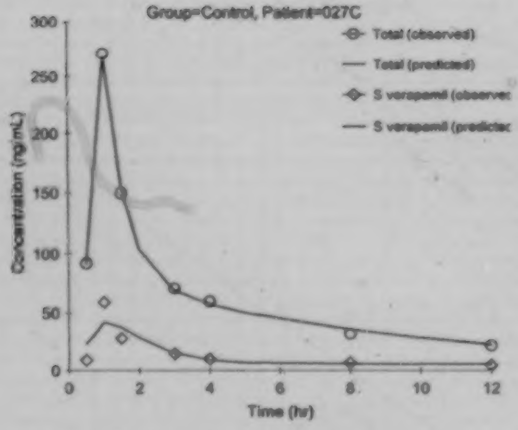
APPENDIX 1

Mean plasma concentrations and mean changes in PR interval were used in analyzing the relationship between pharmacokinetics and pharmacodynamics in this study. A trend was observed towards higher plasma concentrations and lower changes in PR interval in the non-infliximab treated RA group compared with the control group, although these differences were not statistically significant. Nevertheless, reduced drug effect in the presence of the same or higher drug concentrations suggest the pharmacokinetic/pharmacodynamic (PK/PD) relationship was altered. Reduced drug response in the presence of inflammation has been reported in patients with RA and in rat models of acute and chronic inflammation. To further examine the PK/PD relationship, we graphed plasma concentrations versus effect (PR interval prolongation). A counter-clockwise hysteresis was evident for some patients and was subsequently collapsed when the data was fitted to a two-compartment model linked to a theoretical effect compartment. Mean plasma concentration and PR interval data fitted well to the PK/PD link models (r^2>0.9). In this appendix, the PK/PD relationship for individual patients is examined.

Plasma concentrations of S-verapamil were very low in some patients following administration of 80 mg of racemic verapamil. For individual patients' PK/PD analysis, therefore, total verapamil (R-verapamil plus S-verapamil) was analyzed in addition to S-verapamil.

Appendix 1-1. Observed vs predicted concentration-time profile for total and S-verapamil fitted to a 2 compartment first-order input and elimination model.

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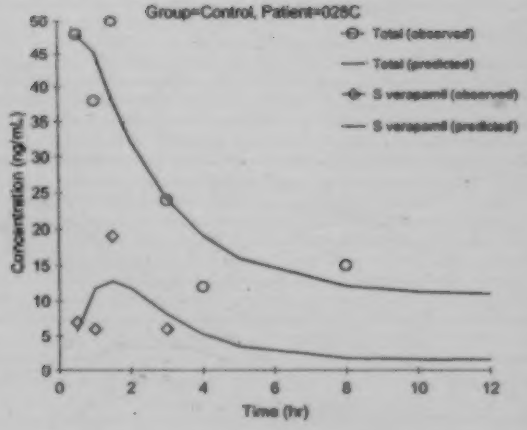
Total (observed)
Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
Time (hr)
0
50
100
150
200
250
300
2
4
6
8
10
12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Total (observed)
Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
Time (hr)
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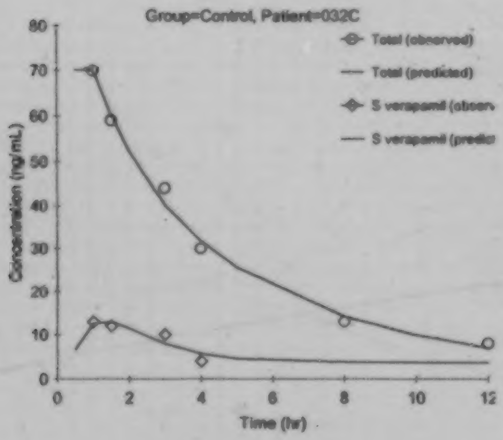
10
15
20
25
30
35
40
45
50
2
4
6
8
10
12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Total (observed)
Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
Time (hr)
0
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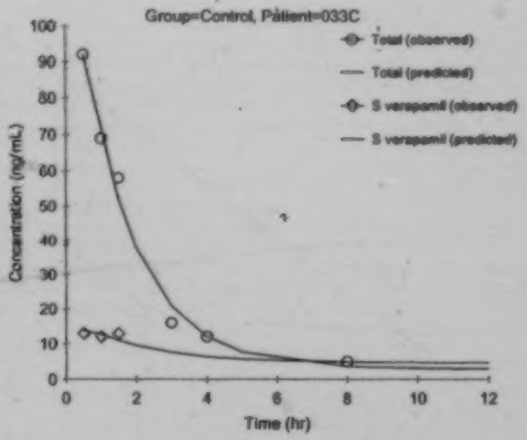
20
30
40
50
60
70
80
2
4
6
8
10
12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Total (observed)
Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
Time (hr)
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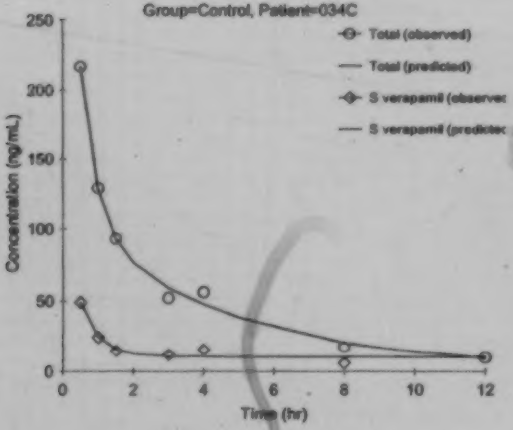
20
30
40
50
60
70
80
90
100
2
4
6
8
10
12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Total (observed)
Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
Time (hr)
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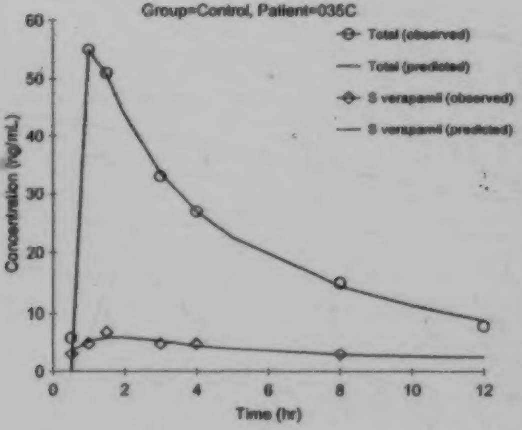
100
150
200
250
2
4
6
8
10
12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Total (observed)
Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
Time (hr)
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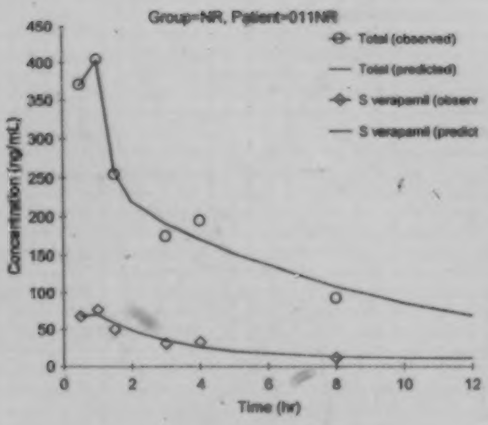
20
30
40
50
60
2
4
6
8
10
12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Total (predicted)
S verapamil (observ
S verapamil (predict
Concentration (ng/mL)
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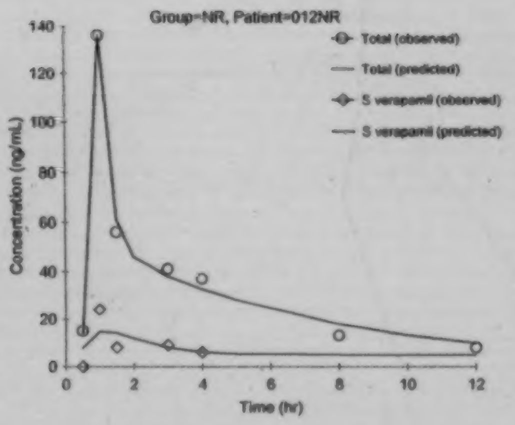
150
200
250
300
350
400
450
Time (hr)
0
2
4
6
8
10
12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Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
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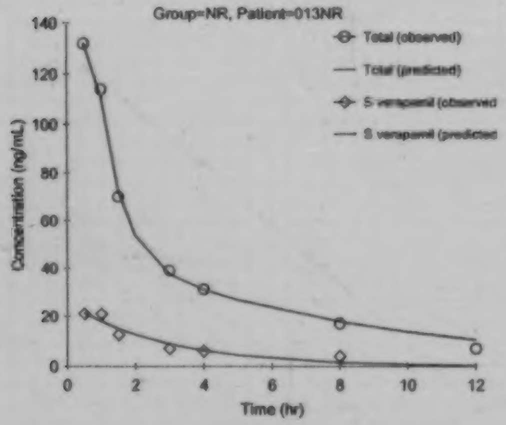
60
80
100
120
140
Time (hr)
0
2
4
6
8
10
12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Total (predicted)
S verapamil (observed
S verapamil (predicted
Concentration (ng/mL)
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80
100
120
140
Time (hr)
0
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8
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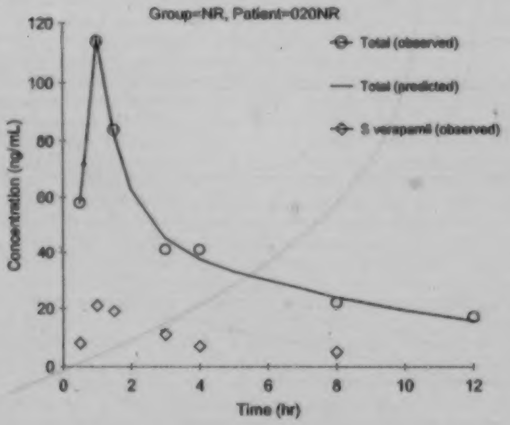
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Total (predicted)
S verapamil (observed)
Concentration (ng/mL)
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60
80
100
120
Time (hr)
0
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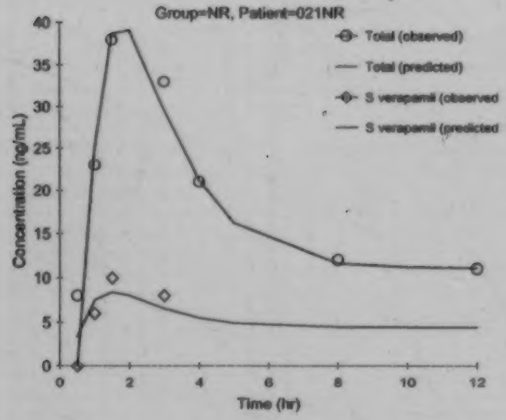
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Total (predicted)
S verapamil (observed
S verapamil (predicted
Concentration (ng/mL)
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Time (hr)
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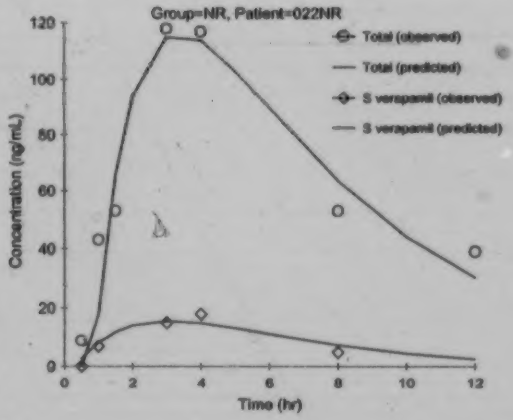
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Total (observed)
Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
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Time (hr)
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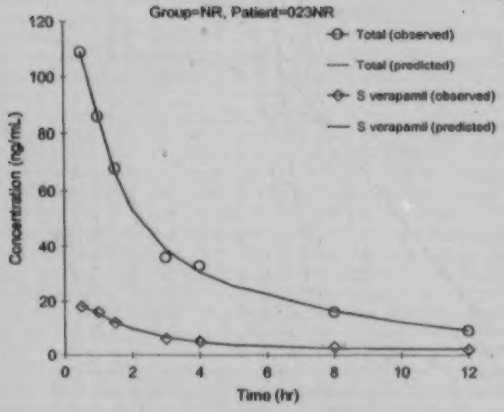
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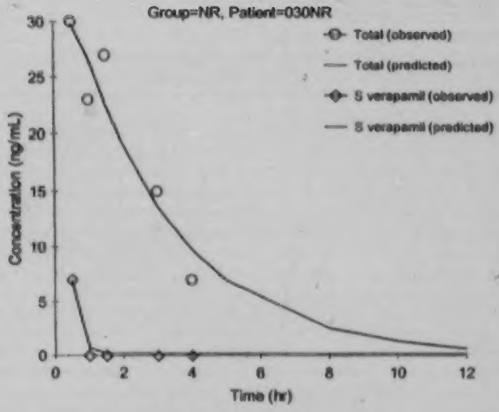
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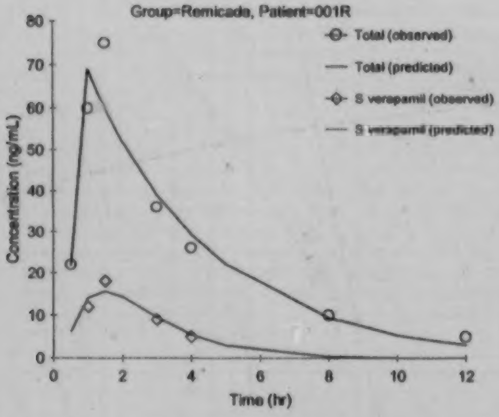
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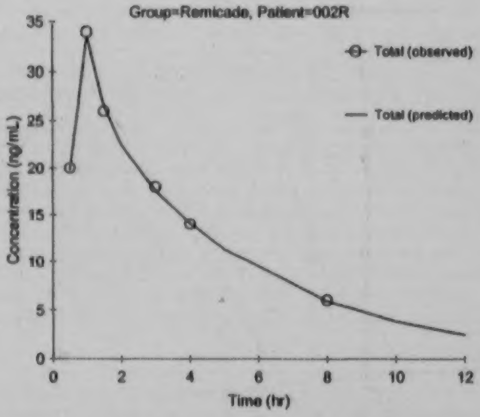
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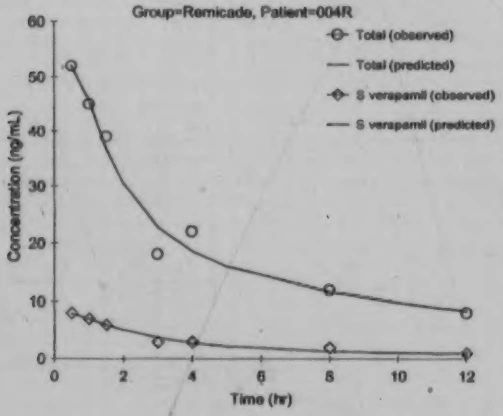
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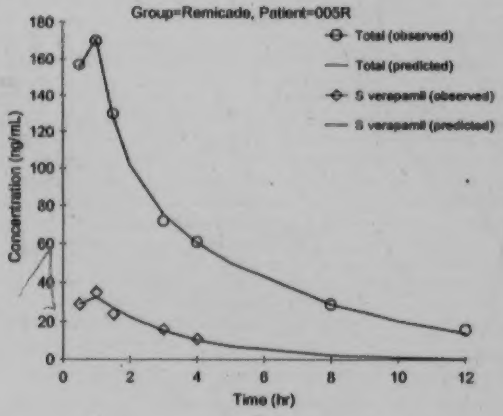
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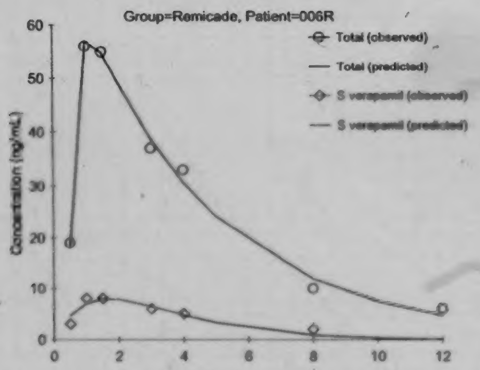
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Total (predicted)
S verapamil (observed)
S verapamil (predicted)
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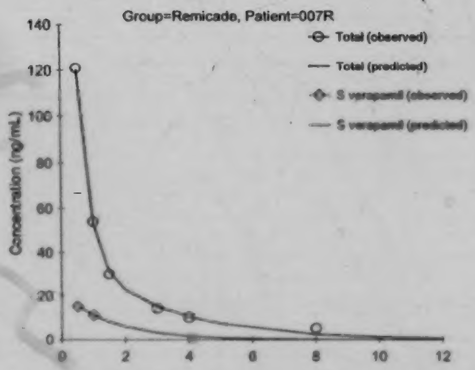
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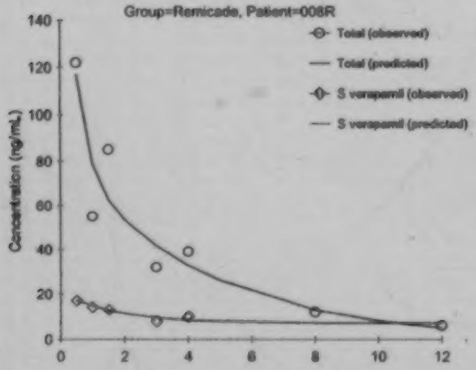
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S verapamil (predicted)
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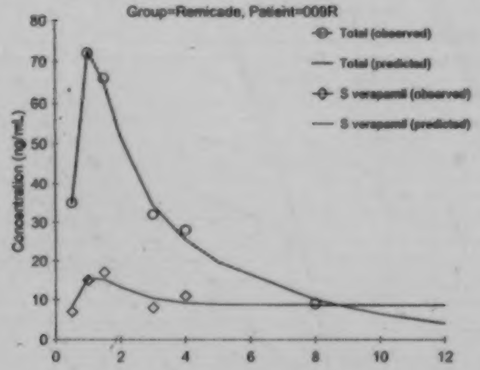
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Total (predicted)
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S verapamil (predicted)
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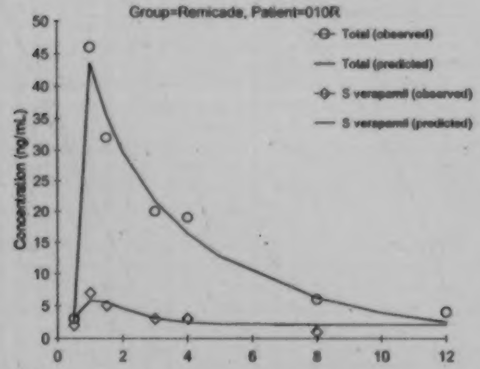
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S verapamil (predicted)
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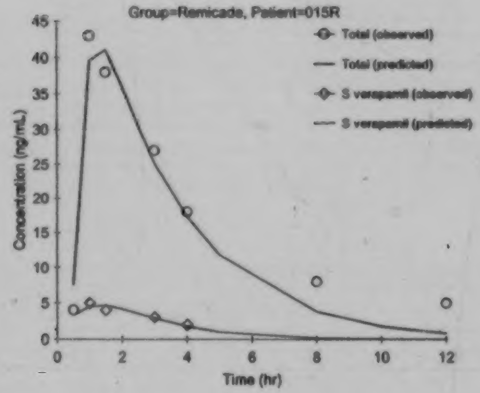
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Total (predicted)
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S verapamil (predicted)
Concentration (ng/mL)
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45
0
2
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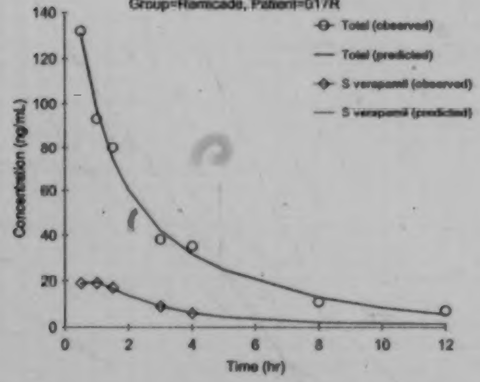
6
8
10
12
Time (hr)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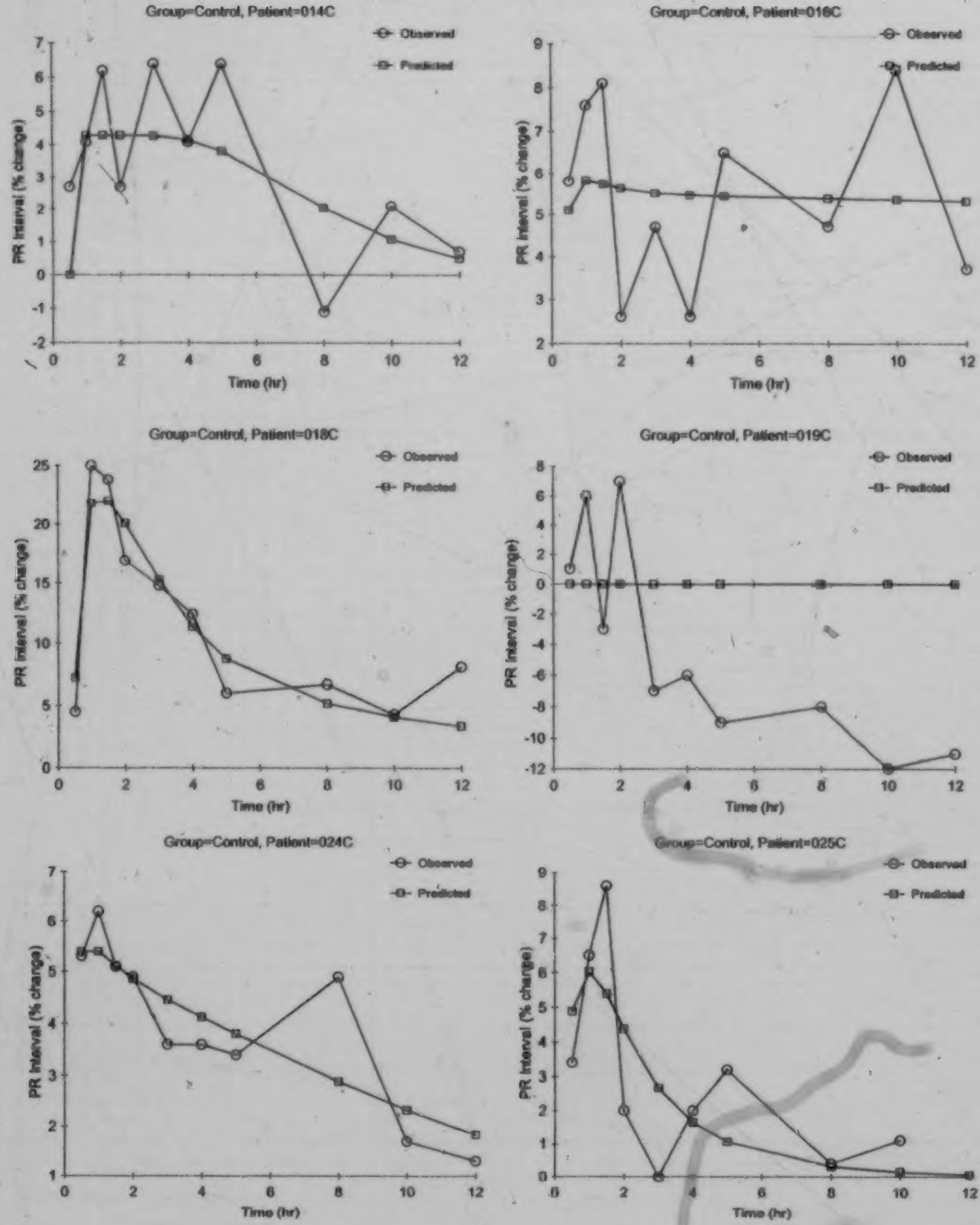
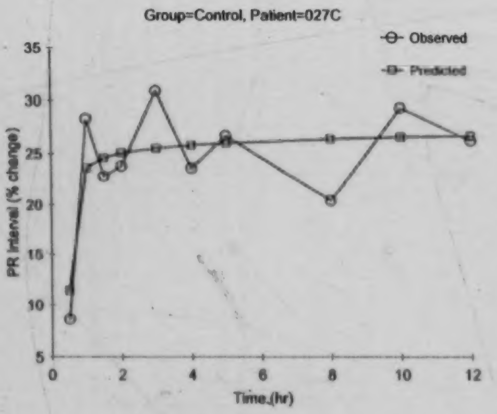
Total (predicted)
S verapamil (observed)
S verapamil (predicted)
Concentration (ng/mL)
0
20
40
60
80
100
120
140
0
2
4
6
8
10
12
Time (hr)

Appendix 1-2. Observed vs predicted percent change in PR interval fitted to a sigmoidal Emax model.

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Observed
Predicted
PR Interval (% change)
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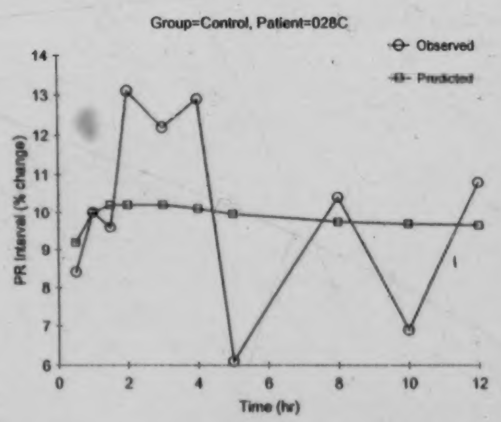
30
25
20
15
10
5
0
2
4
6
8
10
12
Time (hr)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Observed
Predicted
PR Interval (% change)
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13
12
11
10
9
8
7
6
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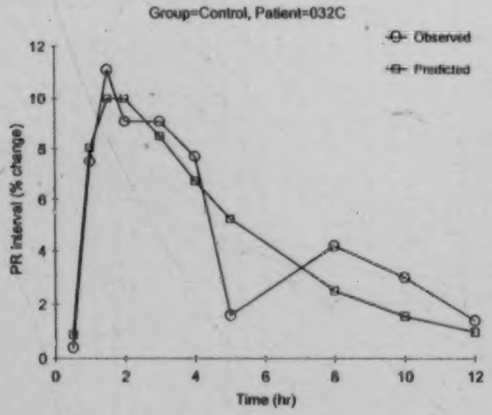
2
4
6
8
10
12
Time (hr)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Observed
Predicted
PR Interval (% change)
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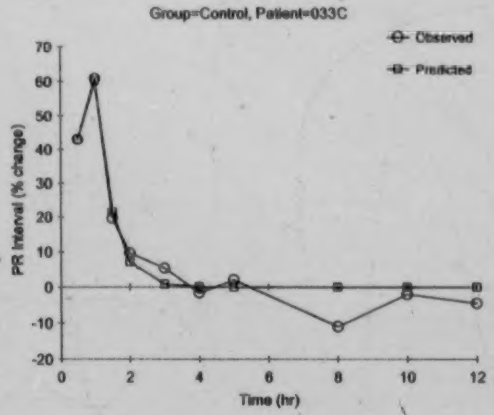
6
4
2
0
0
2
4
6
8
10
12
Time (hr)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Observed
Predicted
PR Interval (% change)
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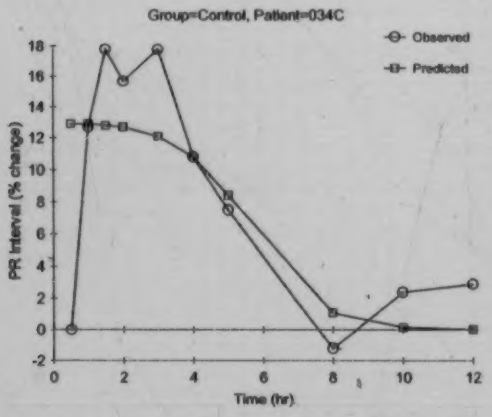
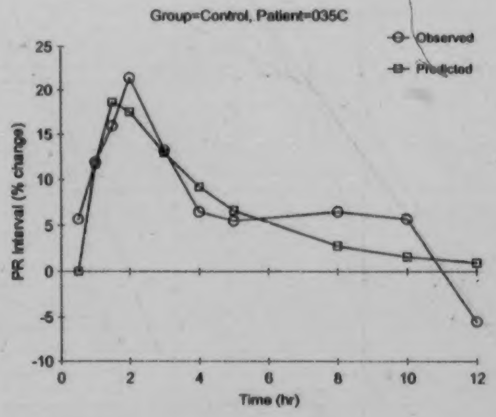
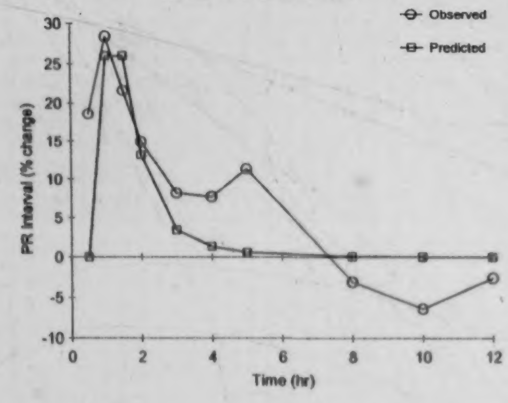
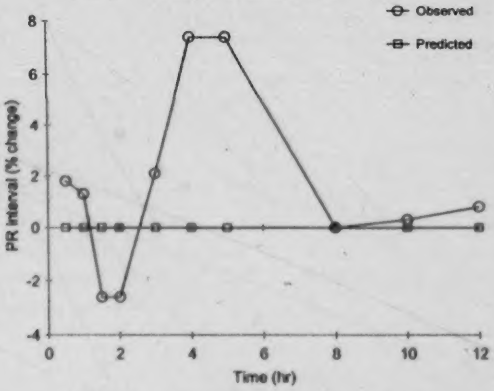
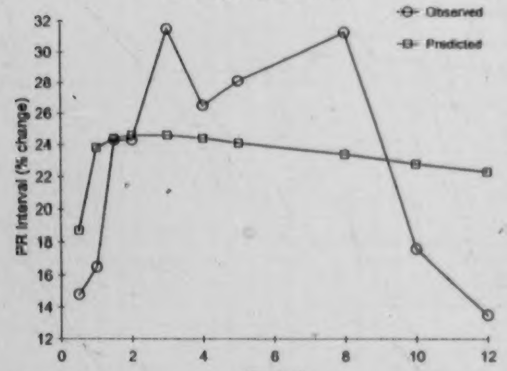
40
30
20
10
0
-10
-20
0
2
4
6
8
10
12
Time (hr)

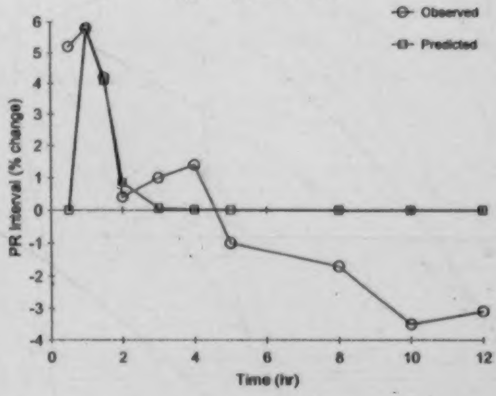

Group=Control, Patient=034C
Observed
Predicted
PR Interval (% change)
18
16
14
12
10
8
6
4
2
0
-2
0
2
4
6
8
10
12
Time (hr)

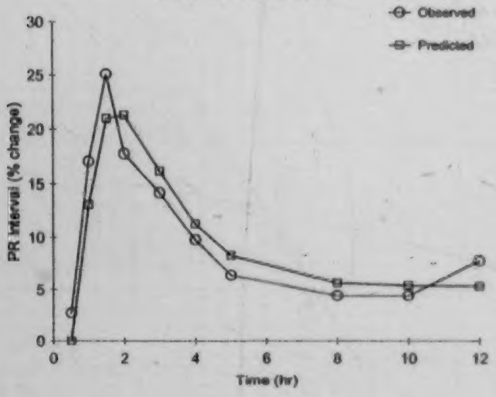

Group=Control, Patient=035C
Observed
Predicted
PR Interval (% change)
25
20
15
10
5
0
-5
-10
0
2
4
6
8
10
12
Time (hr)

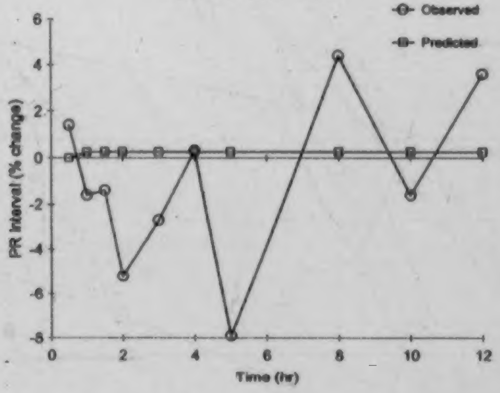

Group=NR, Patient=011NR


Group=NR, Patient=012NR

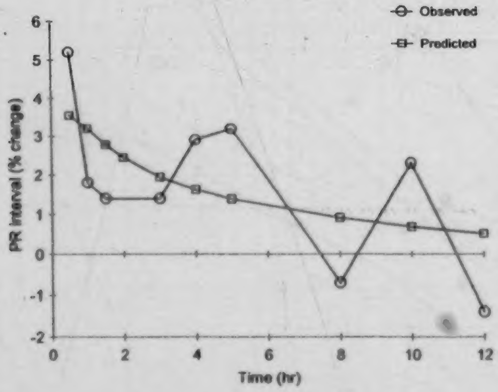

Group=NR, Patient=013NR


Group=NR, Patient=020NR

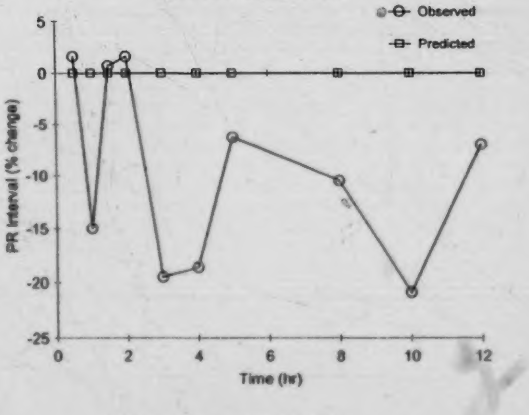

Group=NR, Patient=021NR


Group=NR, Patient=022NR

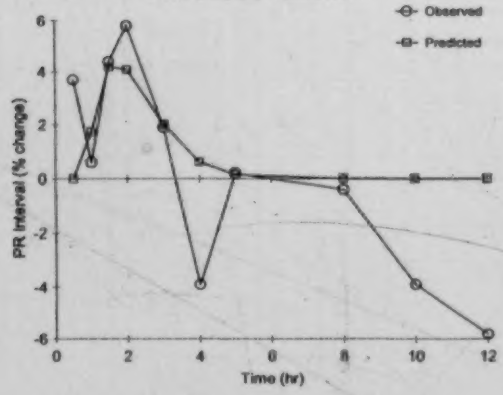
Group=NR, Patient=023NR



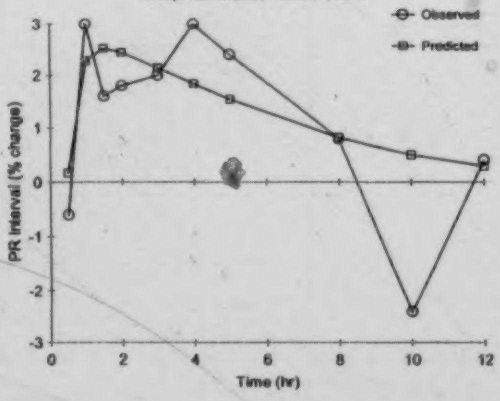
Group=NR, Patient=030NR



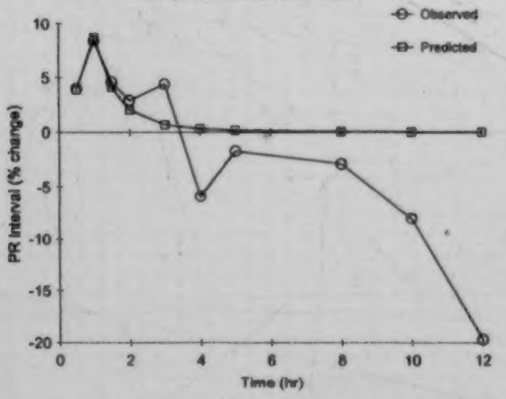
Group=Remicade, Patient=001R



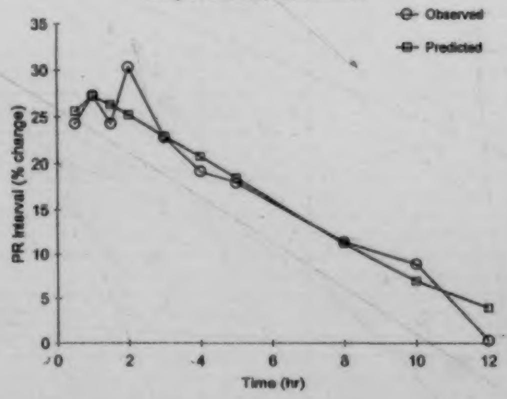
Group=Remicade, Patient=002R

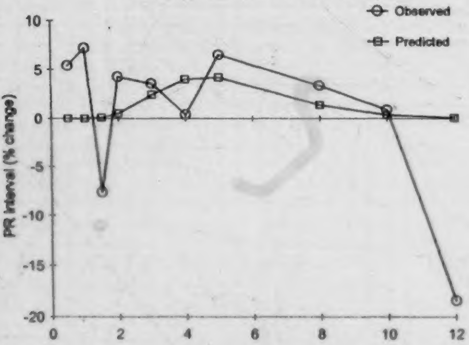


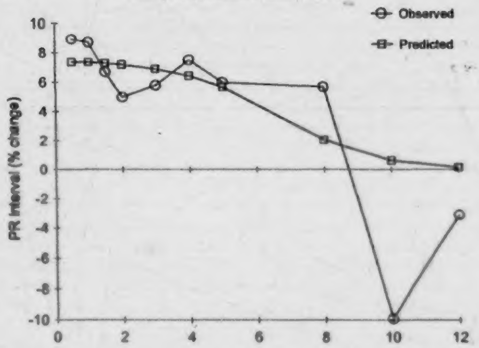
Group=Remicade, Patient=004R

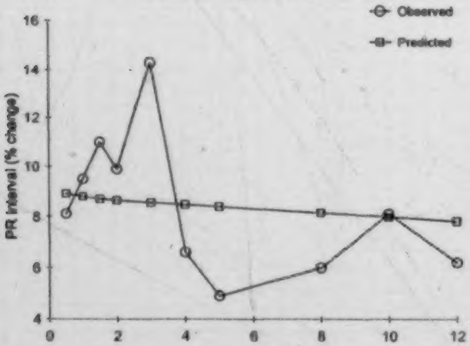


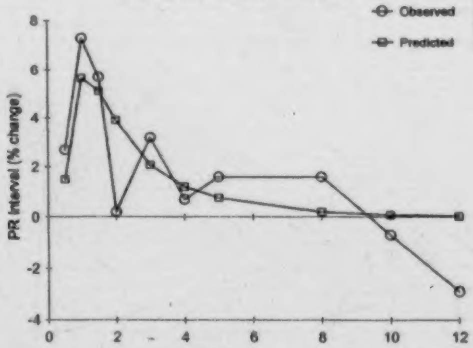
Group=Remicade, Patient=005R

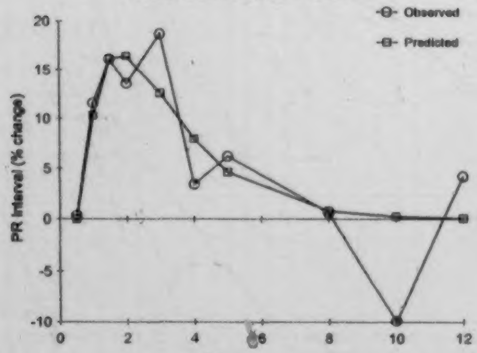


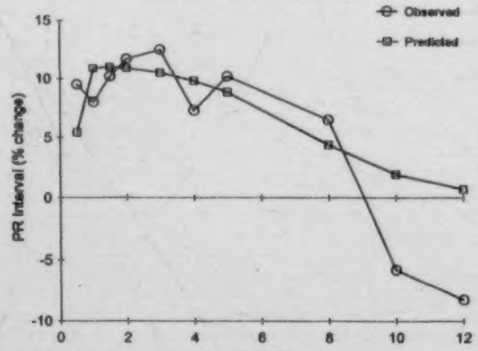

Group=Remicade, Patient=006R

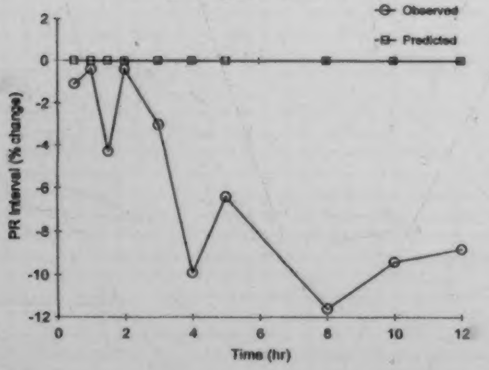

Group=Remicade, Patient=007R


Group=Remicade, Patient=008R

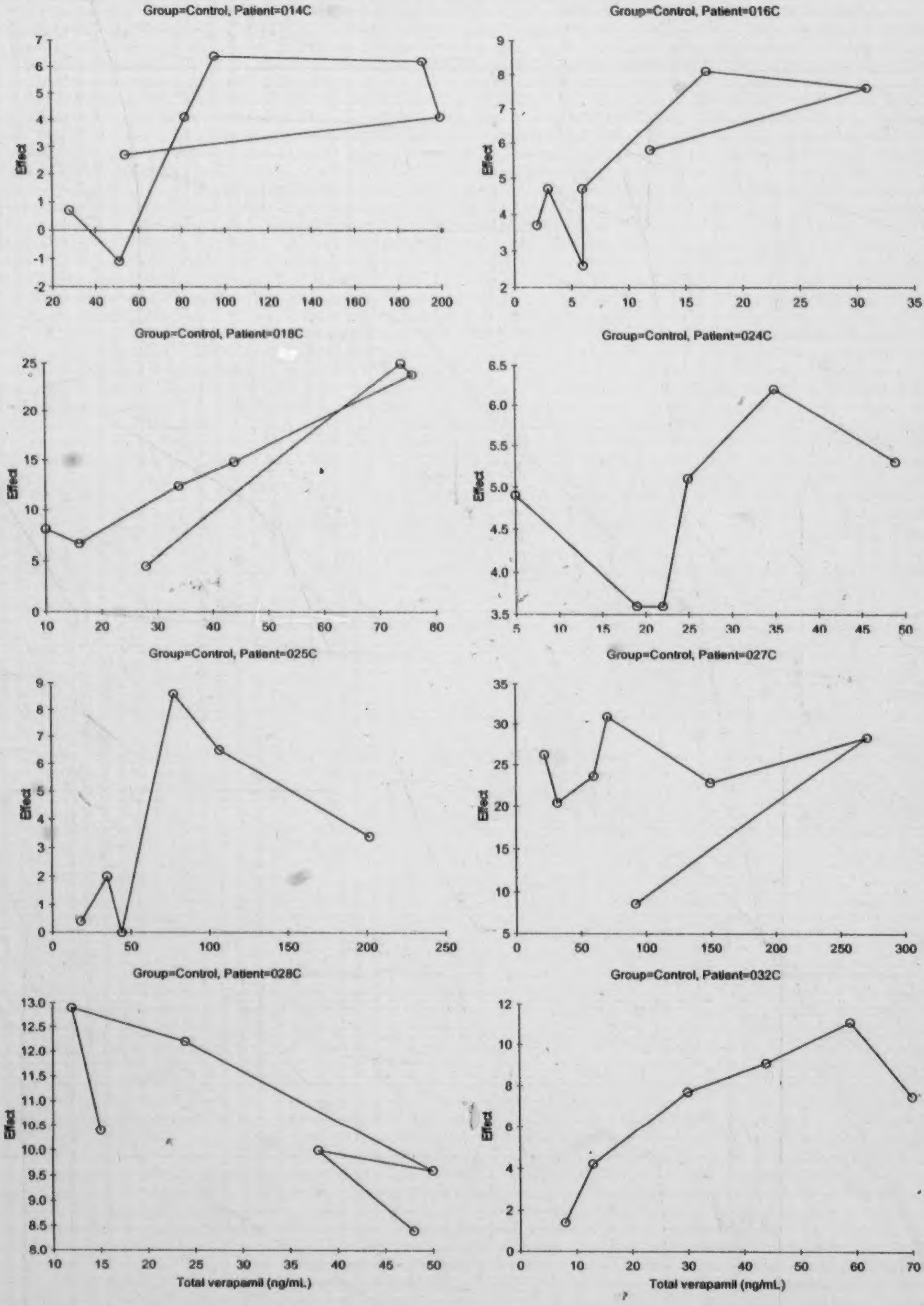

Group=Remicade, Patient=009R

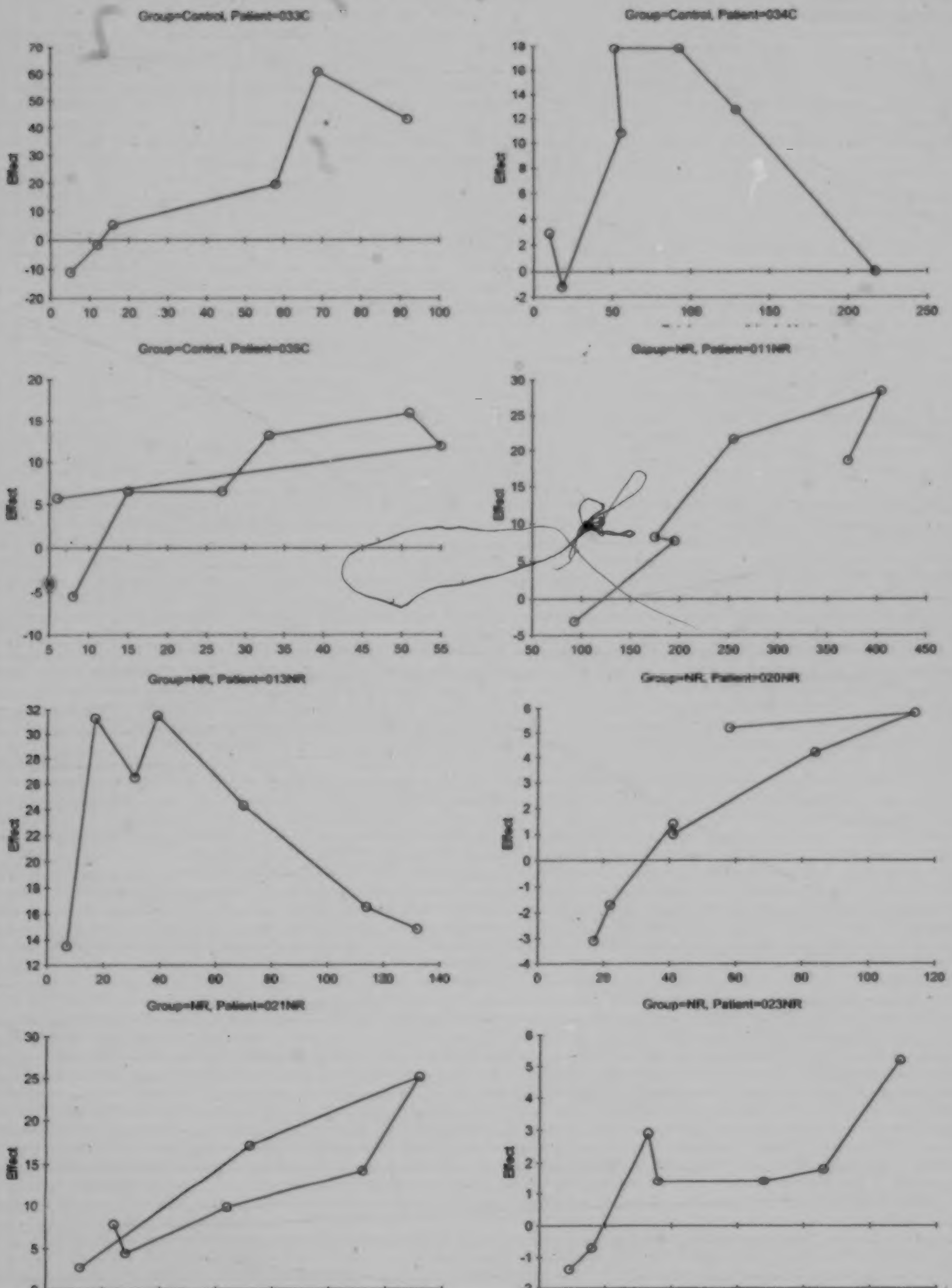

Group=Remicade, Patient=010R


Group=Remicade, Patient=015R

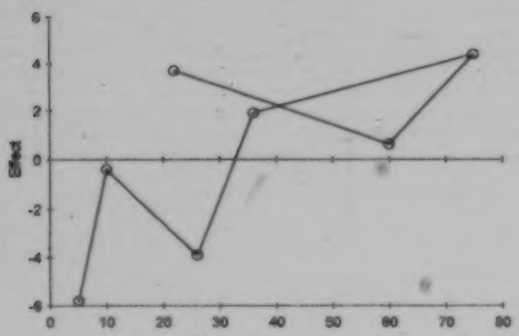

Group=Remicade, Patient=017R

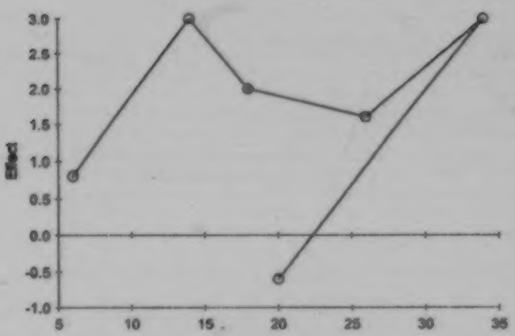
Appendix 1-3. Observed total concentration vs observed effect.

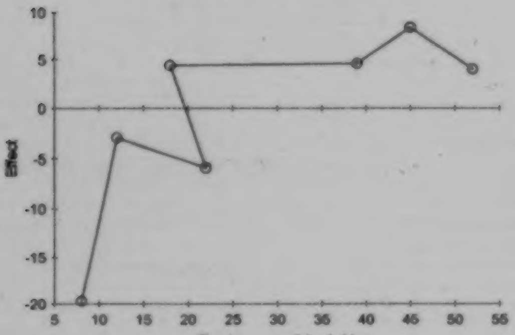


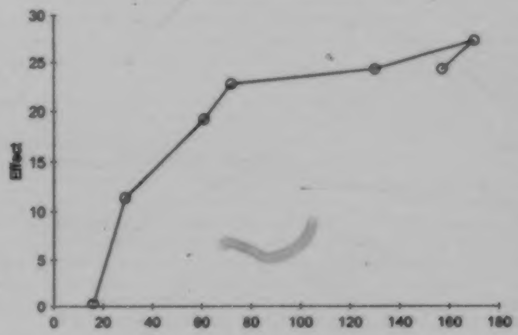


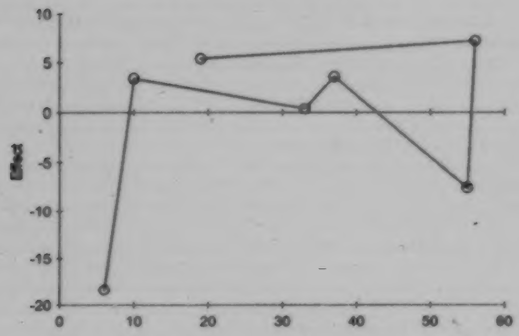
Group=Control, Patient=033C
Effect
70
60
50
40
30
20
10
0
-10
-20
0 10 20 30 40 50 60 70 80 90 100
Group=Control, Patient=034C
Effect
18
16
14
12
10
8
6
4
2
0
-2
0 50 100 150 200 250
Group=Control, Patient=035C
Effect
20
15
10
5
0
-5
-10
5 10 15 20 25 30 35 40 45 50 55
Group=NR, Patient=011NR
Effect
30
25
20
15
10
5
0
-5
50 100 150 200 250 300 350 400 450
Group=NR, Patient=013NR
Effect
32
30
28
26
24
22
20
18
16
14
12
0 20 40 60 80 100 120 140
Group=NR, Patient=020NR
Effect
6
5
4
3
2
1
0
-1
-2
-3
-4
0 20 40 60 80 100 120
Group=NR, Patient=021NR
Effect
30
25
20
15
10
5
0
5 10 15 20 25 30 35 40
Total verapamil (ng/mL)
Group=NR, Patient=023NR
Effect
6
5
4
3
2
1
0
-1
-2
0 20 40 60 80 100 120
Total verapamil (ng/mL)

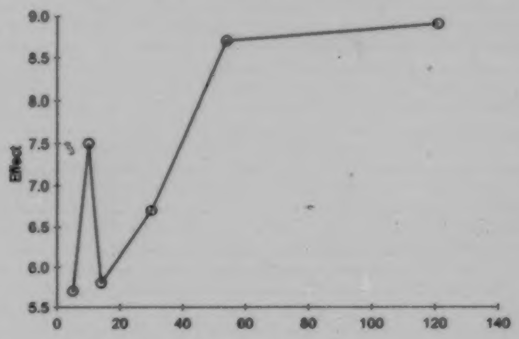

Group=Remicade, Patient=001R

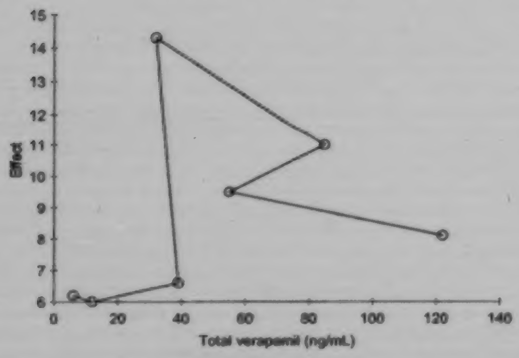

Group=Remicade, Patient=002R

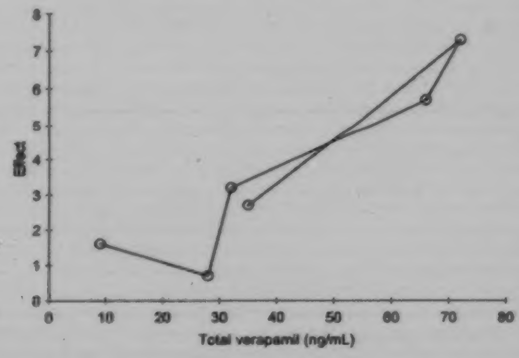

Group=Remicade, Patient=004R

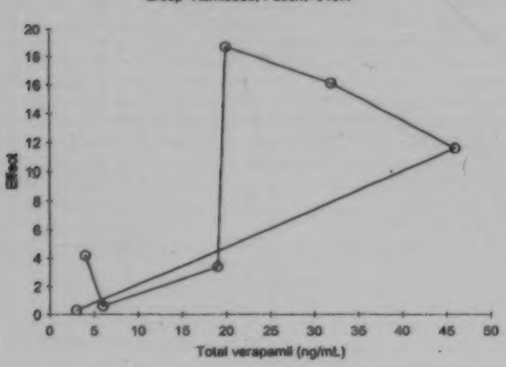

Group=Remicade, Patient=005R


Group=Remicade, Patient=006R

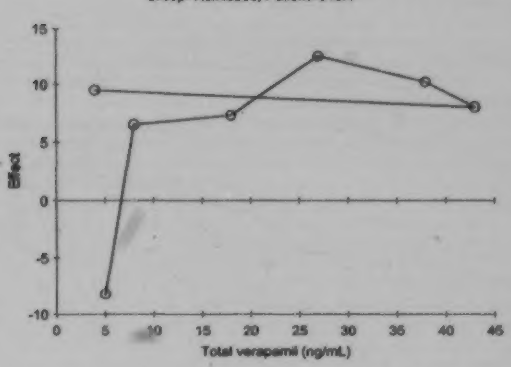

Group=Remicade, Patient=007R


Group=Remicade, Patient=008R


Group=Remicade, Patient=009R

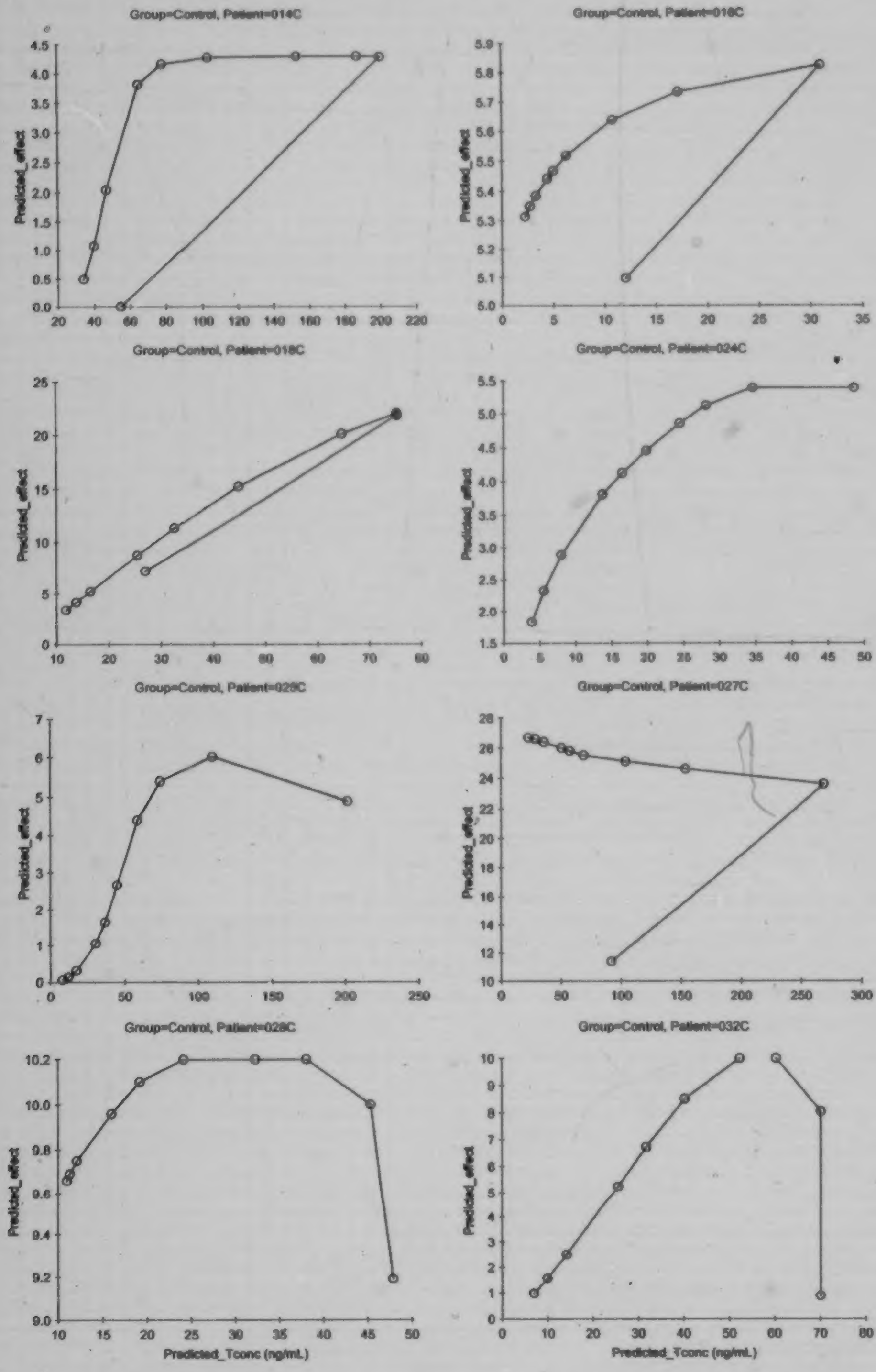


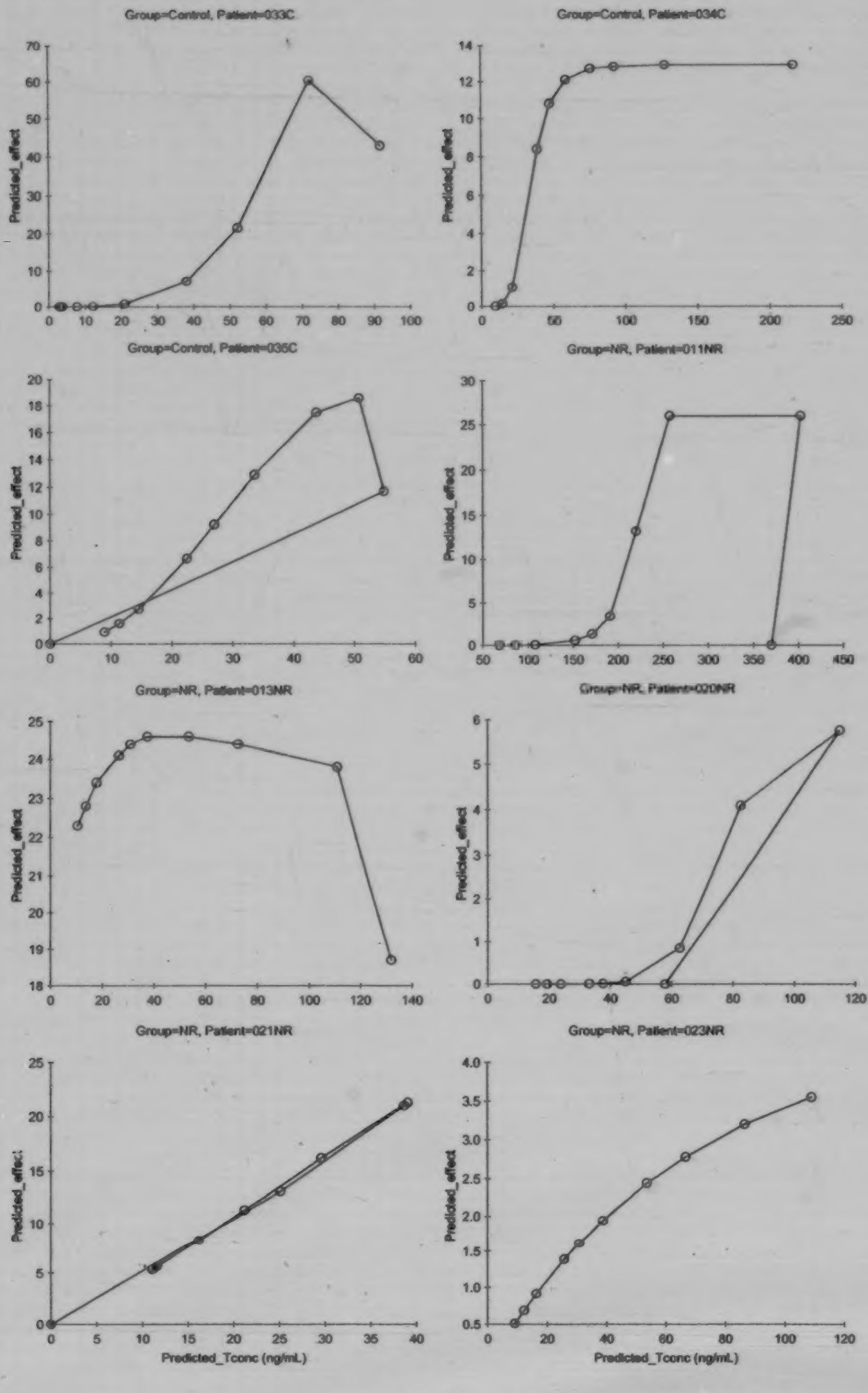
Group=Remicade, Patient=010R



Group=Remicade, Patient=015R

Appendix 1-4. Predicted total concentration vs predicted effect.





Group=Control, Patient=033C

Group=Control, Patient=034C

Group=Control, Patient=035C

Group=NR, Patient=011NR

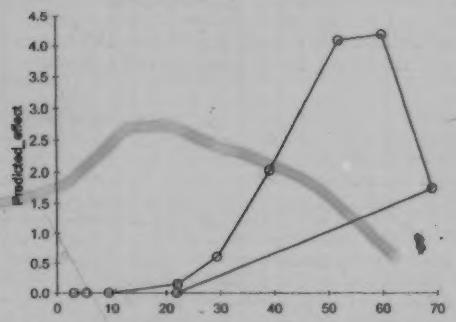
Group=NR, Patient=013NR

Group=NR, Patient=020NR

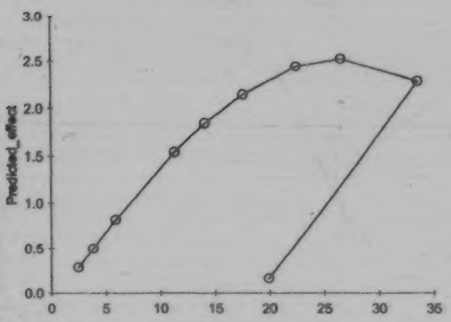
Group=NR, Patient=021NR

Group=NR, Patient=023NR

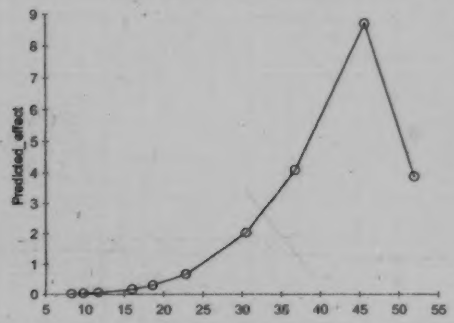
Group=Remicade, Patient=001R



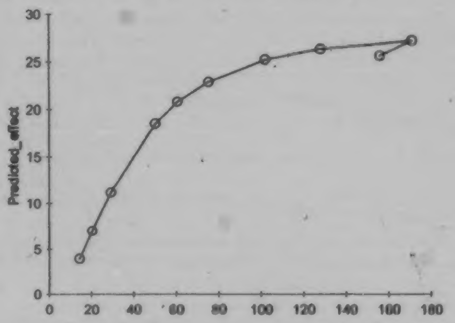
Group=Remicade, Patient=002R



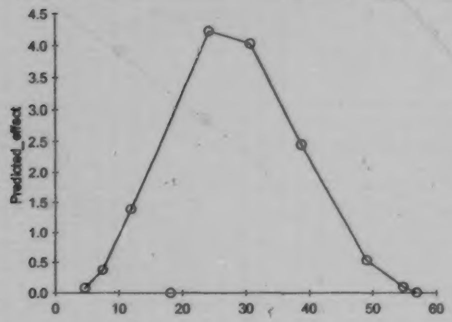
Group=Remicade, Patient=004R



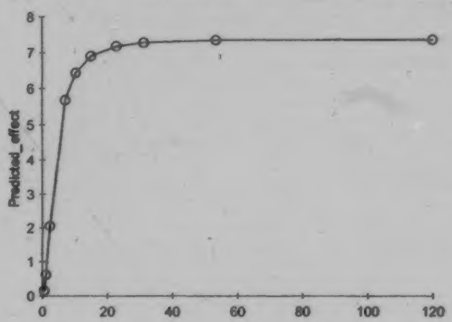
Group=Remicade, Patient=005R



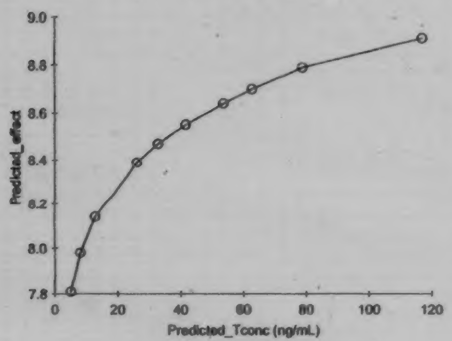
Group=Remicade, Patient=006R



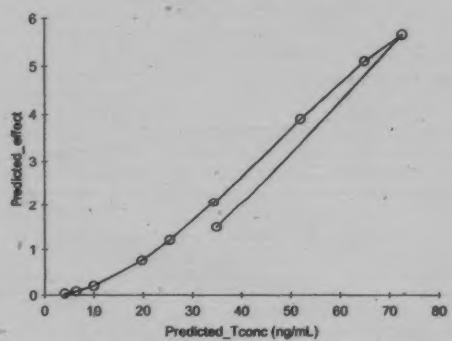
Group=Remicade, Patient=007R

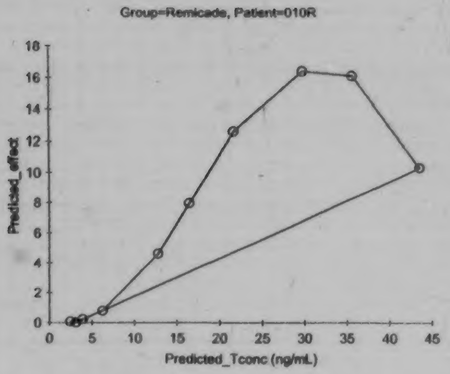


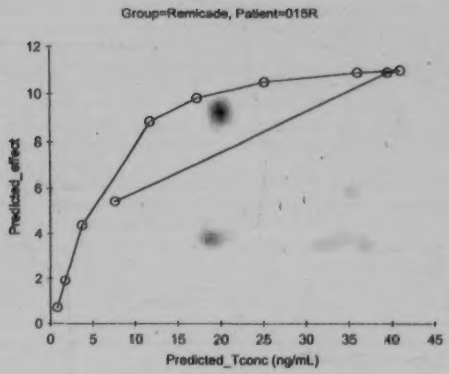
Group=Remicade, Patient=008R



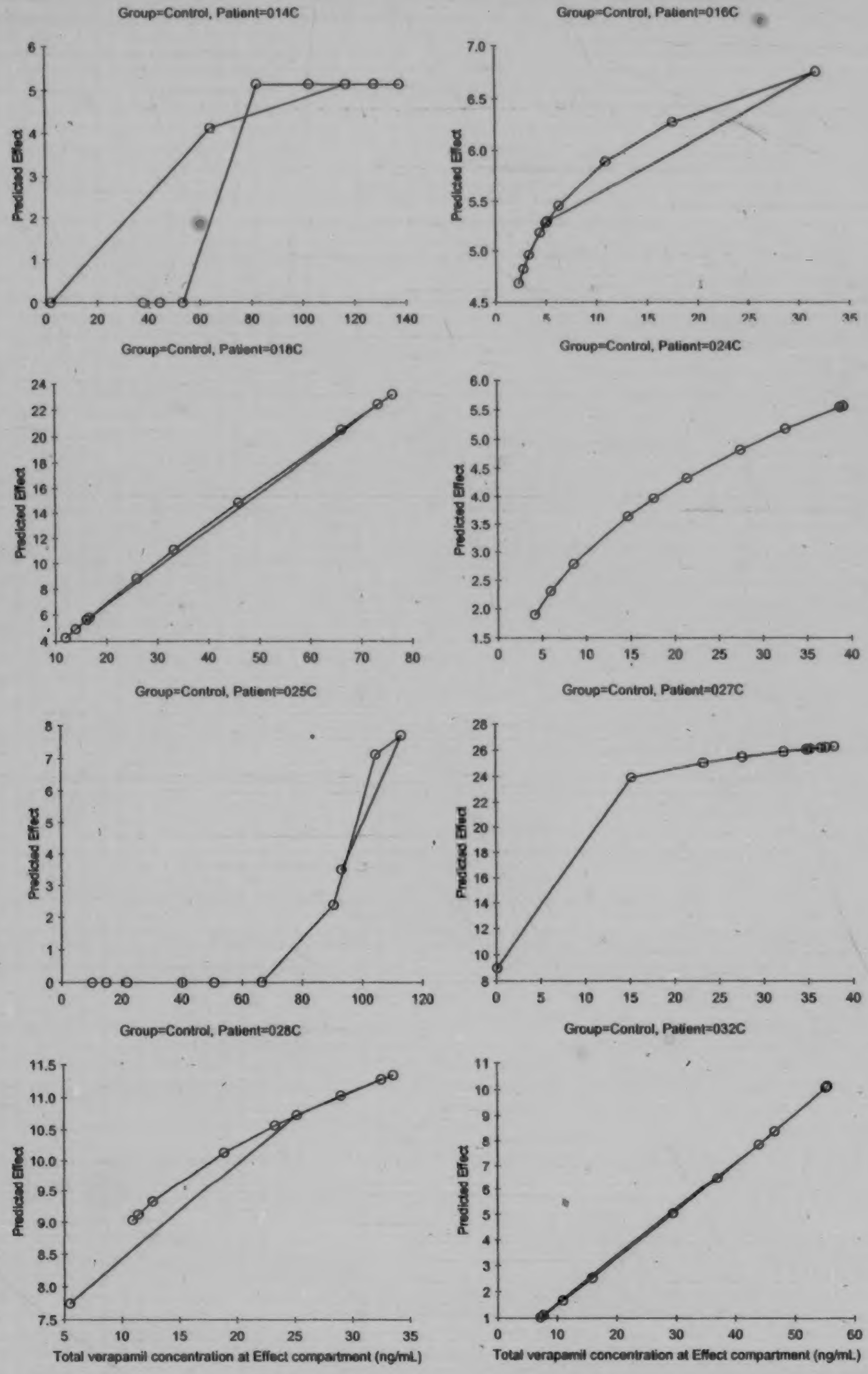
Group=Remicade, Patient=009R

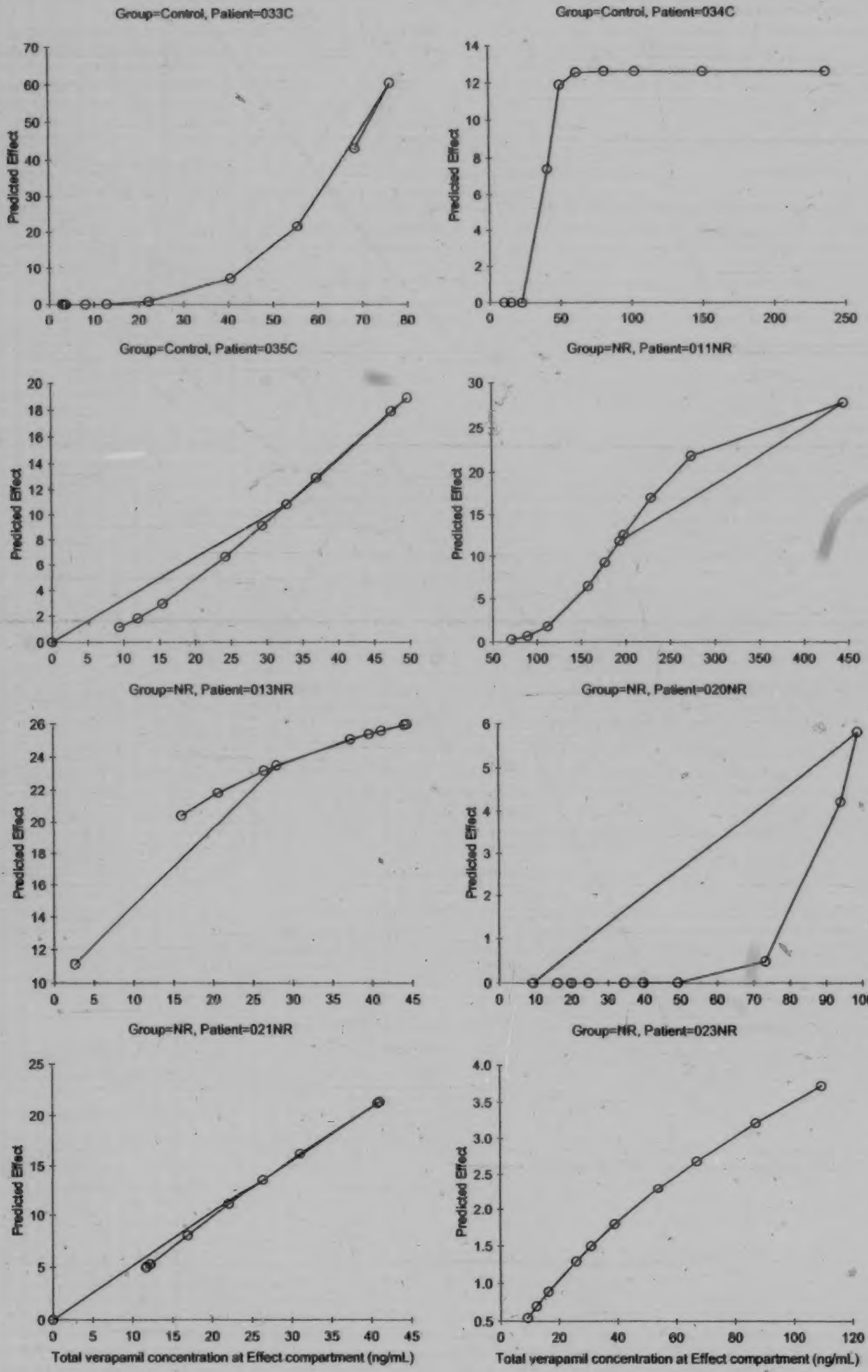


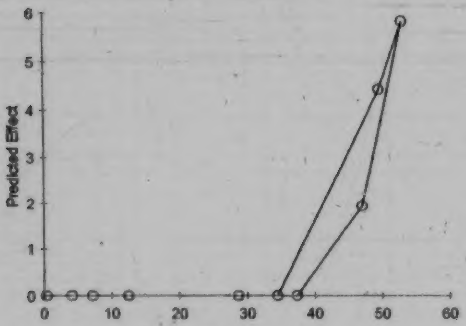

Group=Remicade, Patient=010R
Predicted_effect
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25
30
35
40
45
Predicted_Tconc (ng/mL)


Group=Remicade, Patient=015R
Predicted_effect
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
40
45
Predicted_Tconc (ng/mL)

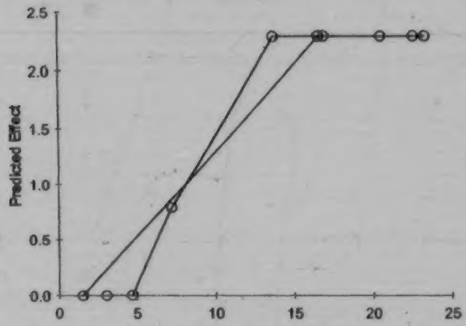
Appendix 1-5. Predicted total concentration at effect compartment vs predicted effect.



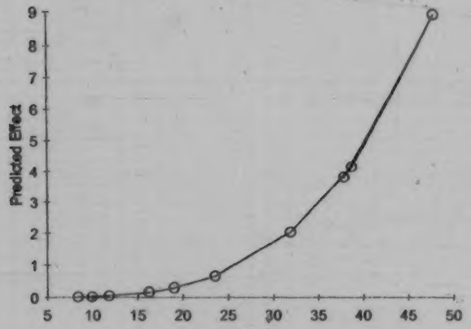

Group=Control, Patient=033C
Group=Control, Patient=034C
Group=Control, Patient=035C
Group=NR, Patient=011NR
Group=NR, Patient=013NR
Group=NR, Patient=020NR
Group=NR, Patient=021NR
Group=NR, Patient=023NR
Predicted Effect
Total verapamil concentration at Effect compartment (ng/mL)
0
10
20
30
40
50
60
70
80
100
150
200
250
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0



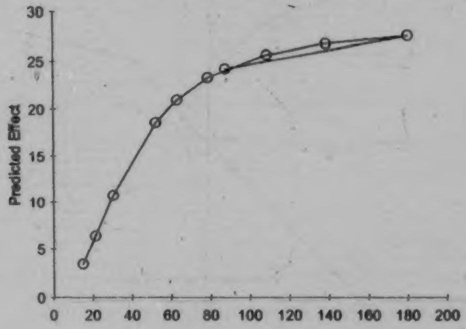
Group=Remicade, Patient=001R



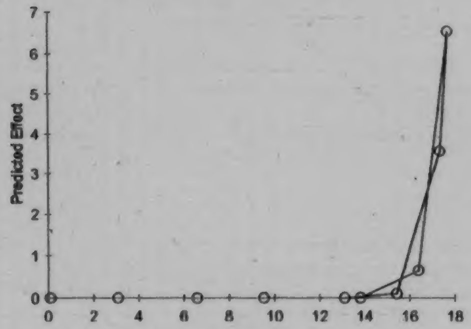
Group=Remicade, Patient=002R



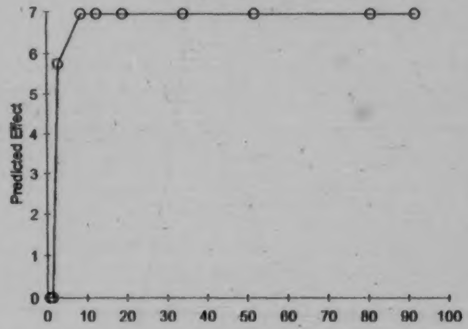
Group=Remicade, Patient=004R



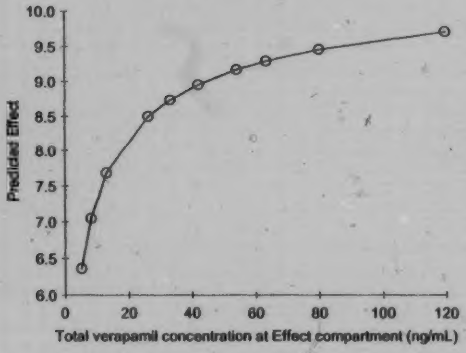
Group=Remicade, Patient=005R



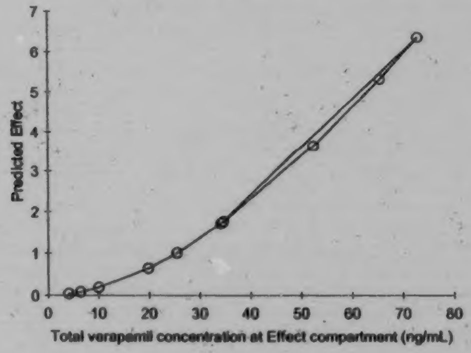
Group=Remicade, Patient=006R



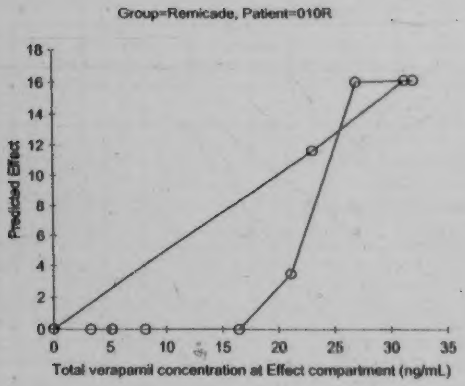
Group=Remicade, Patient=007R

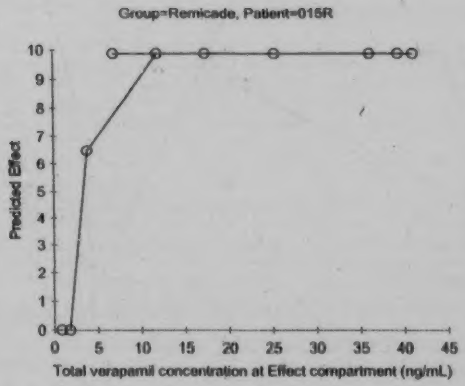


Group=Remicade, Patient=008R

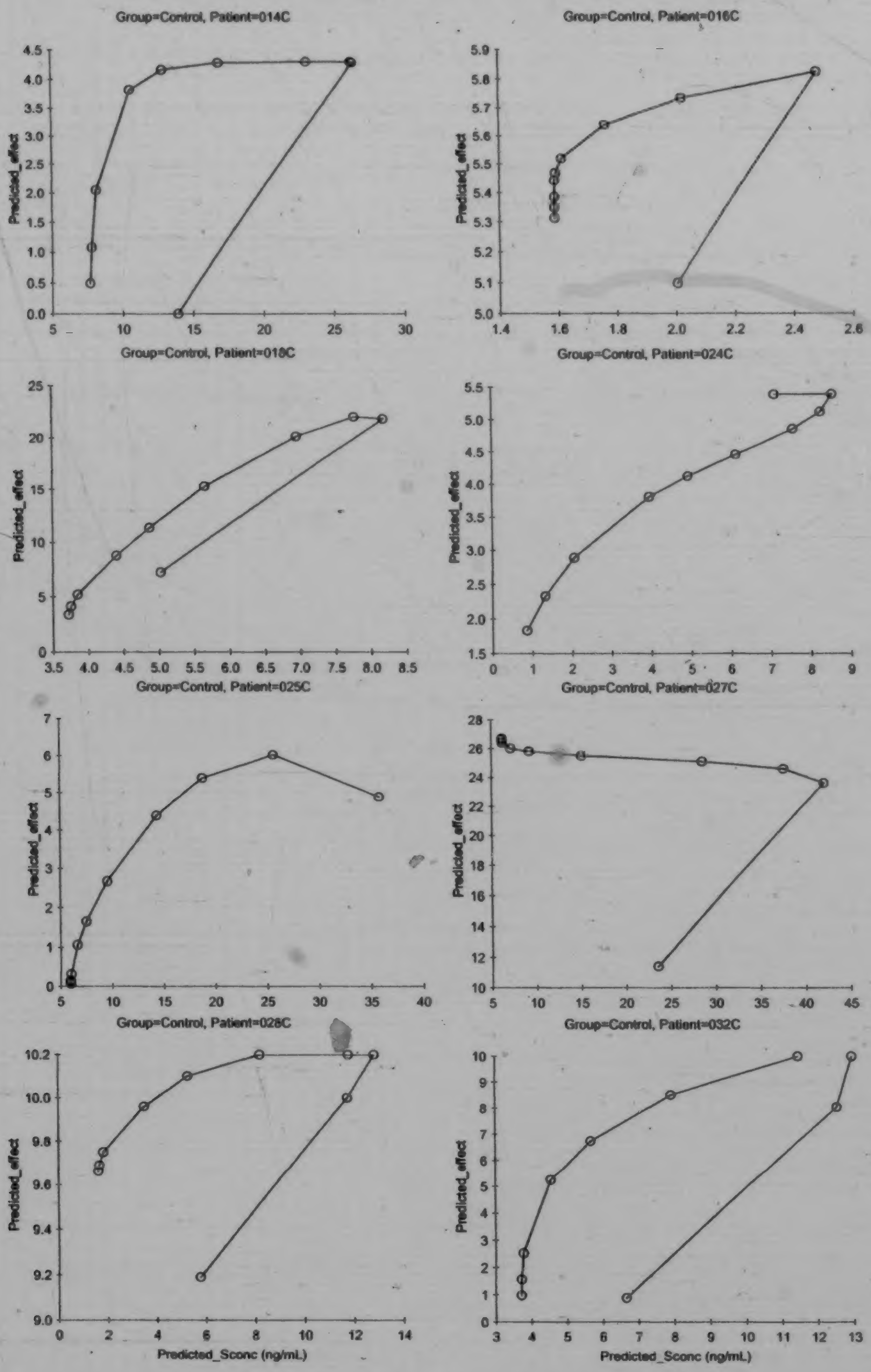


Group=Remicade, Patient=009R


Group=Remicade, Patient=010R


Group=Remicade, Patient=015R

Appendix 1-6. Predicted S-verapamil plasma concentration vs predicted effect.



Group=Control, Patient=014C

Group=Control, Patient=016C

Group=Control, Patient=018C

Group=Control, Patient=024C

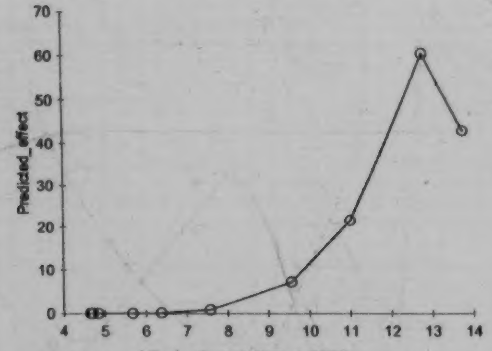
Group=Control, Patient=025C

Group=Control, Patient=027C

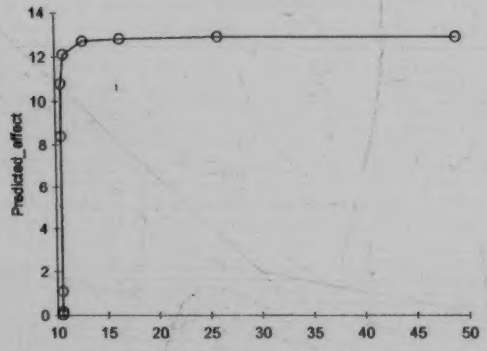
Group=Control, Patient=028C

Group=Control, Patient=032C

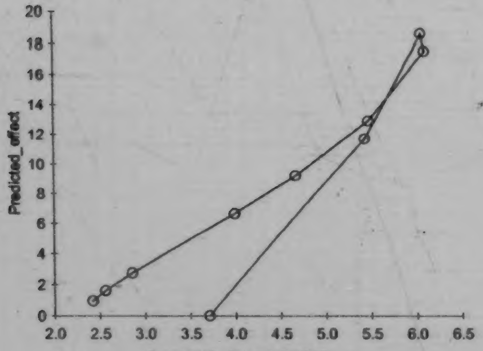
Group=Control, Patient=033C



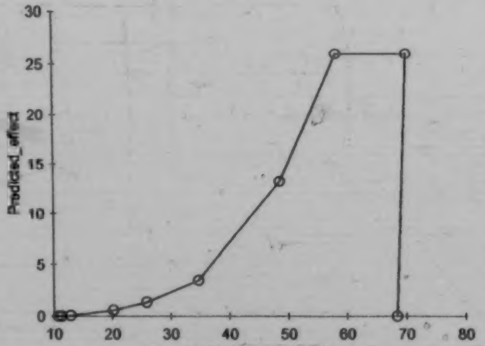
Group=Control, Patient=034C



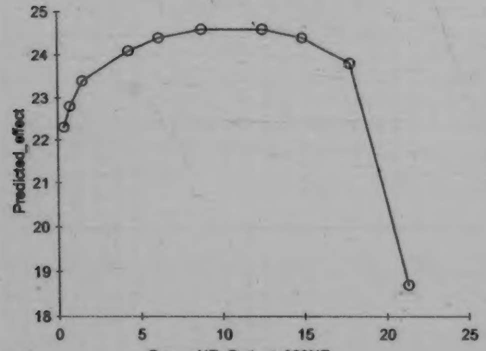
Group=Control, Patient=035C



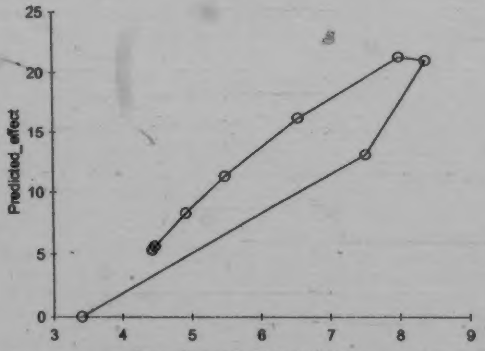
Group=NR, Patient=011NR



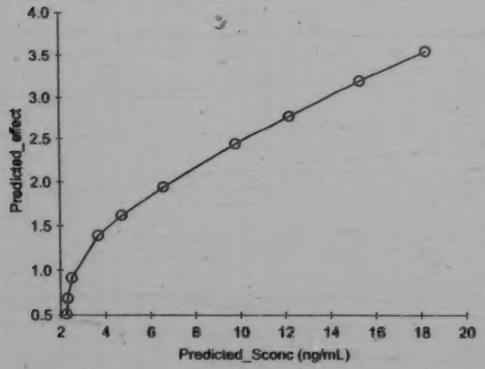
Group=NR, Patient=013NR



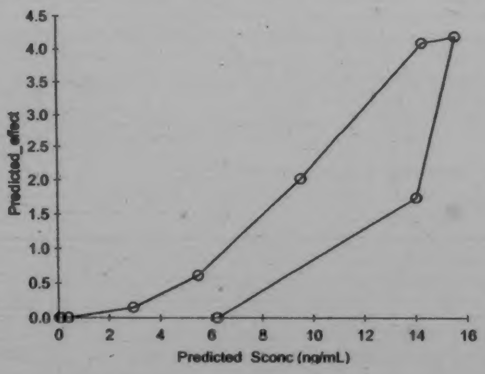
Group=NR, Patient=021NR

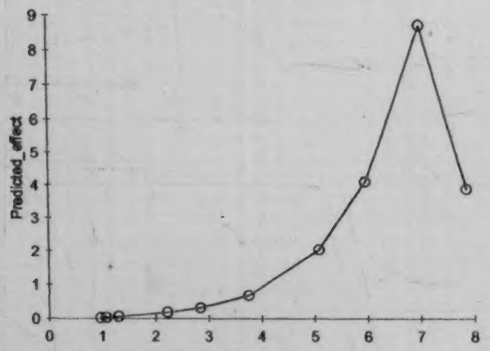


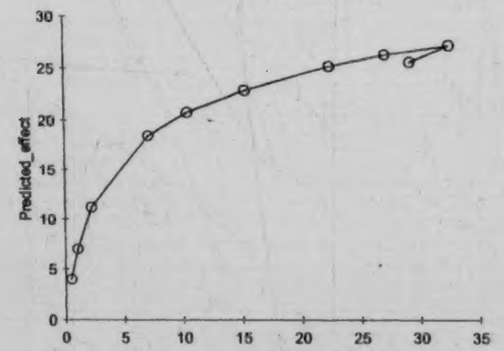
Group=NR, Patient=023NR

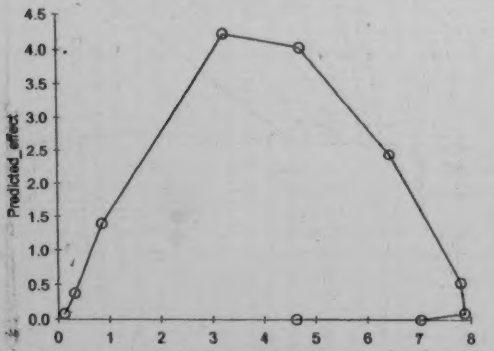


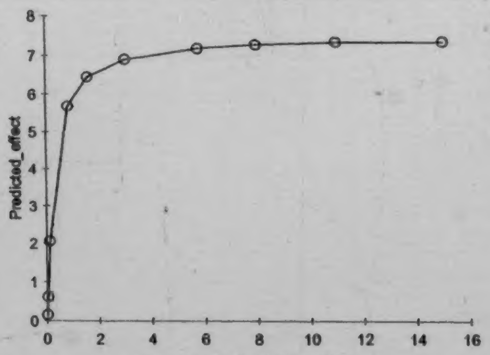
Group=Remicade, Patient=001R

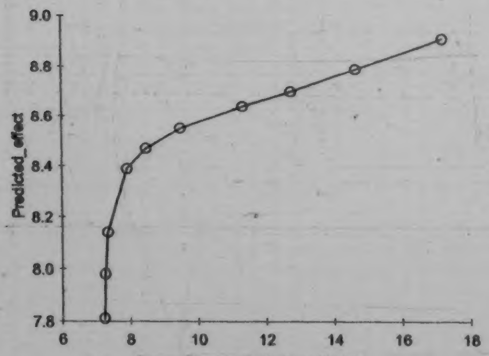


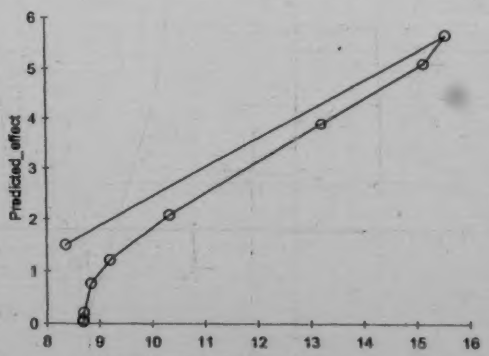

Group=Remicade, Patient=004R
Predicted_effect
0
1
2
3
4
5
6
7
8
9
0
1
2
3
4
5
6
7
8

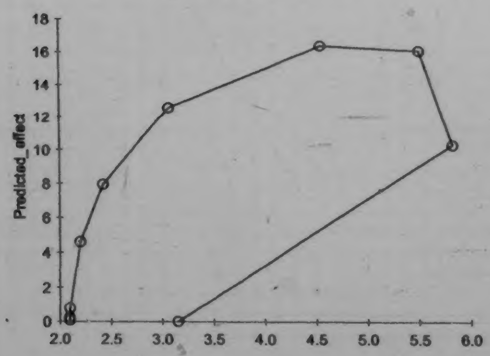

Group=Remicade, Patient=005R
Predicted_effect
0
5
10
15
20
25
30
0
5
10
15
20
25
30
35

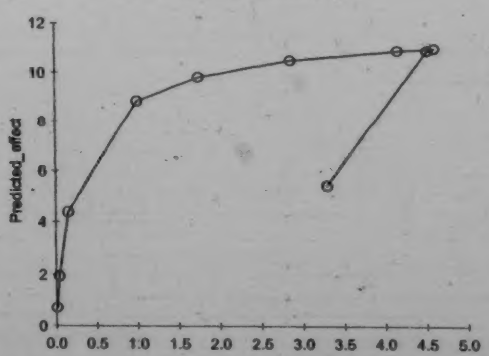

Group=Remicade, Patient=006R
Predicted_effect
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
0
1
2
3
4
5
6
7
8


Group=Remicade, Patient=007R
Predicted_effect
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
16

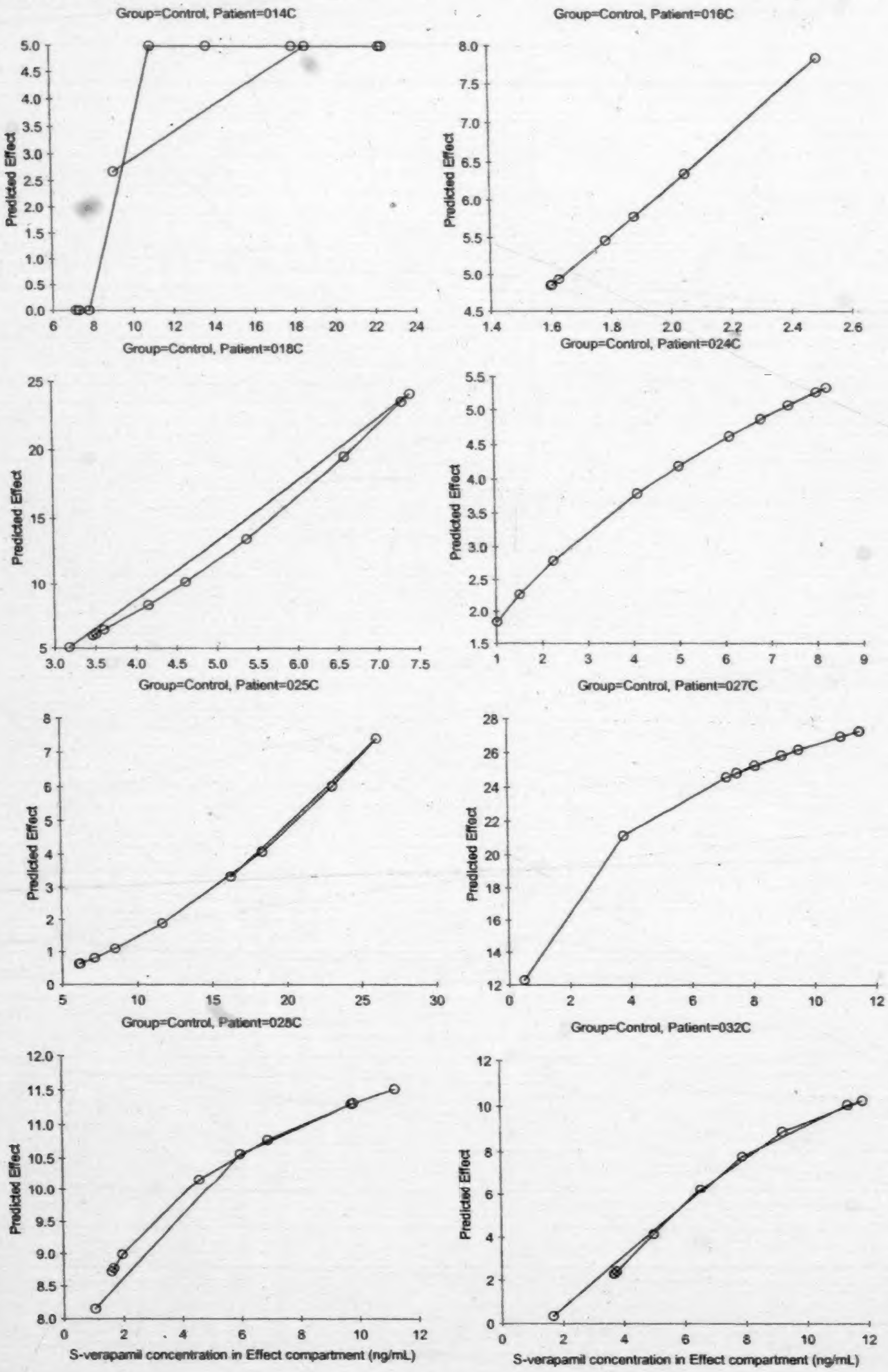

Group=Remicade, Patient=008R
Predicted_effect
7.8
8.0
8.2
8.4
8.6
8.8
9.0
6
8
10
12
14
16
18

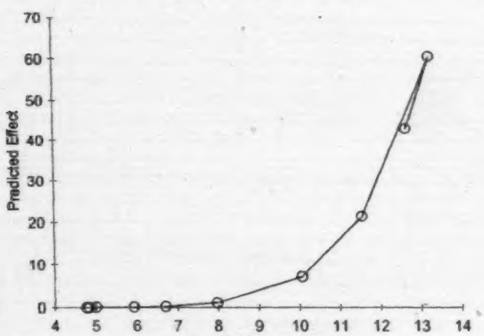

Group=Remicade, Patient=009R
Predicted_effect
0
1
2
3
4
5
6
8
9
10
11
12
13
14
15
16

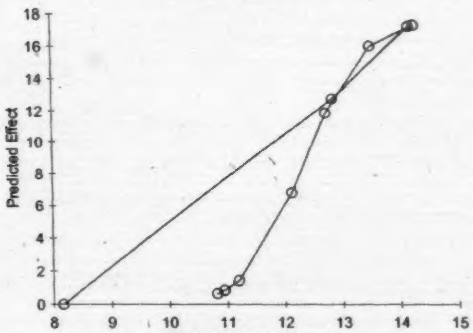

Group=Remicade, Patient=010R
Predicted_effect
0
2
4
6
8
10
12
14
16
18
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0

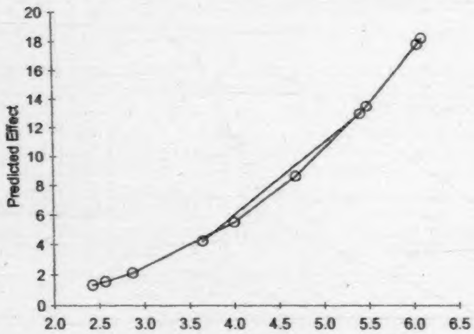

Group=Remicade, Patient=015R
Predicted_effect
0
2
4
6
8
10
12
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0

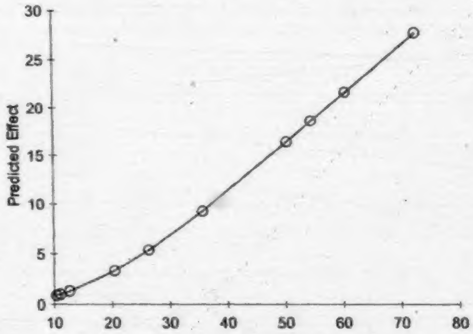
Appendix 1-7. Predicted S-verapamil concentration at effect compartment vs predicted effect.

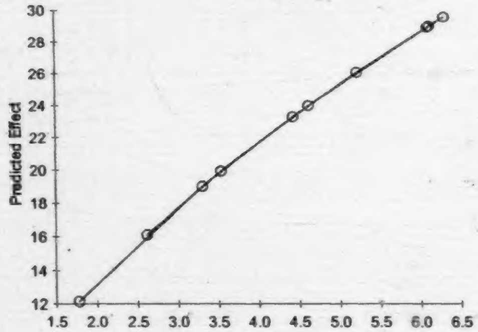


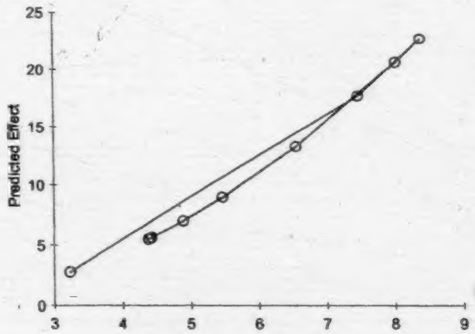

Group=Control, Patient=033C

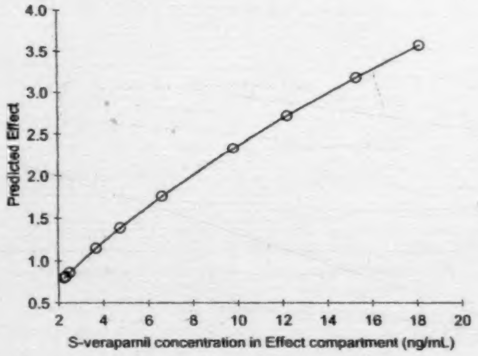

Group=Control, Patient=034C

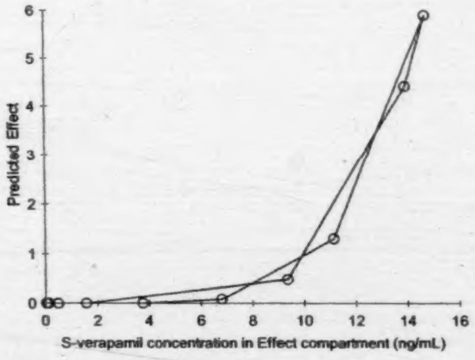

Group=Control, Patient=035C


Group=NR, Patient=011NR

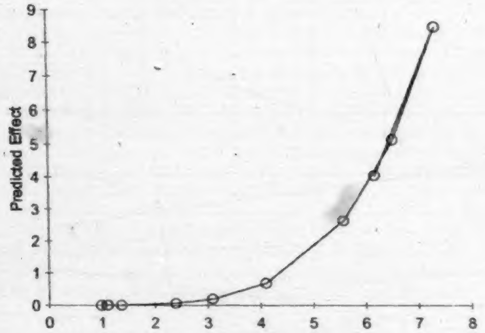

Group=NR, Patient=013NR


Group=NR, Patient=021NR

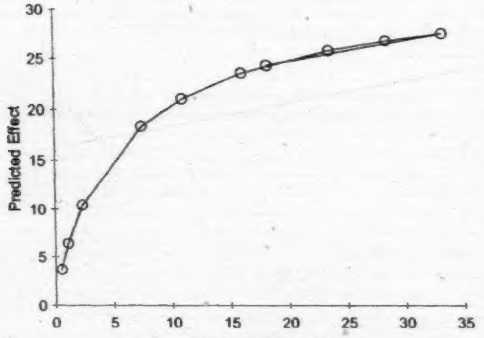

Group=NR, Patient=023NR


Group=Remicade, Patient=001R

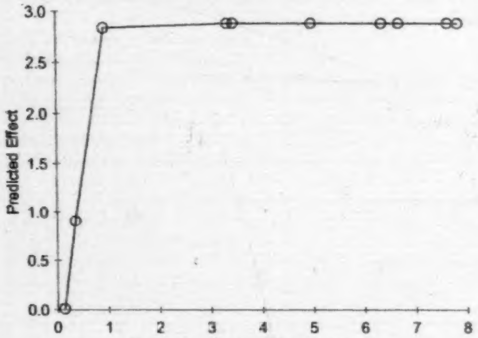
Group=Remicade, Patient=004R



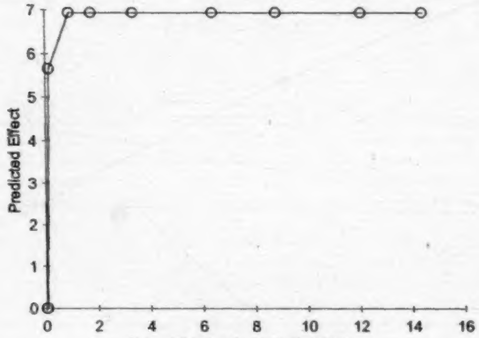
Group=Remicade, Patient=005R



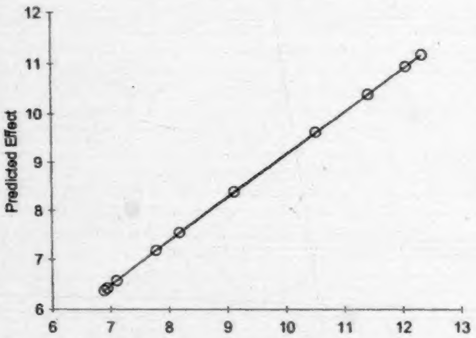
Group=Remicade, Patient=006R



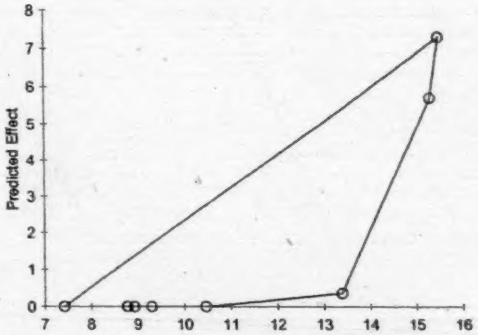
Group=Remicade, Patient=007R



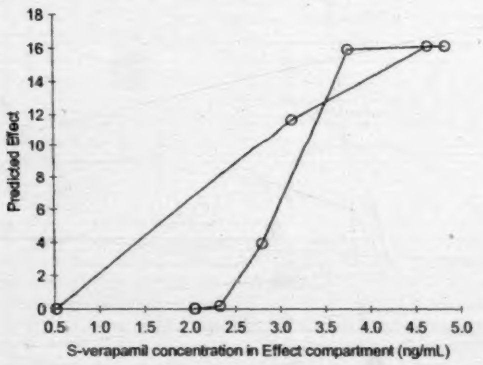
Group=Remicade, Patient=008R



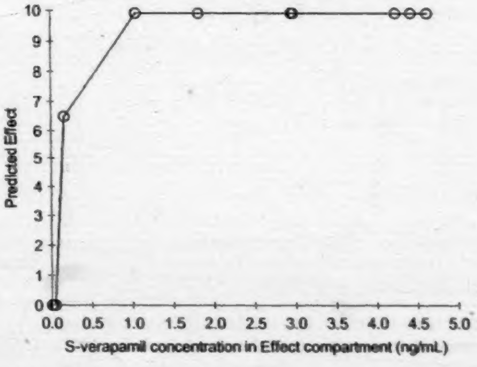
Group=Remicade, Patient=009R



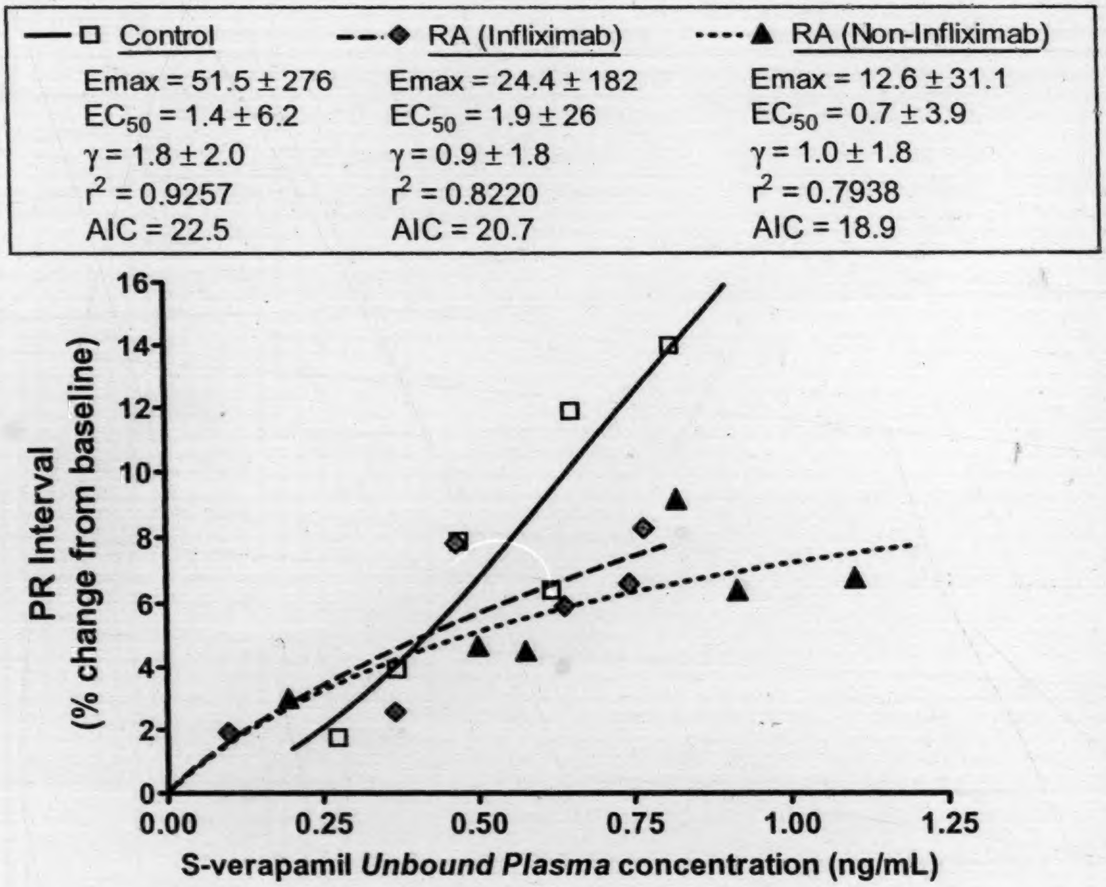
Group=Remicade, Patient=010R



Group=Remicade, Patient=015R



Appendix 2.


Control
RA (Infliximab)
RA (Non-Infliximab)
Emax = 51.5 ± 276
EC50 = 1.4 ± 6.2
γ = 1.8 ± 2.0
r2 = 0.9257
AIC = 22.5
Emax = 24.4 ± 182
EC50 = 1.9 ± 26
γ = 0.9 ± 1.8
r2 = 0.8220
AIC = 20.7
Emax = 12.6 ± 31.1
EC50 = 0.7 ± 3.9
γ = 1.0 ± 1.8
r2 = 0.7938
AIC = 18.9
PR Interval
(% change from baseline)
0
2
4
6
8
10
12
14
16
0.00
0.25
0.50
0.75
1.00
1.25
S-verapamil Unbound Plasma concentration (ng/mL)